UNIVERSITY OF ALBERTA

TRANSCRIPTIONAL REGULATION OF THE MURINE GRANZYME B GENE IN CYTOTOXIC T CELLS

by

CHAROLYN KIM BABICHUK ©

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "THE TRANSCRIPTIONAL REGULATION OF THE MURINE GRANZYME B GENE IN CYTOTOXIC T CELLS" submitted by Charolyn K. Babichuk in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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ABSTRACT

The molecular signals that are involved in cytotoxic T cell activation may be investigated by studying the transcriptional regulation of activation-dependent genes. One such gene is granzyme B, which encodes a protein that is an integral component of the cytolytic machinery. The studies described in this thesis examine the genetic regulatory sequences that are involved in the expression of the murine granzyme B gene.

Potentially important regulatory sequences in the granzyme B promoter were identified by deletion analysis and a 243 bp promoter fragment consistently produced high levels of activity in T cells. Within this fragment, five distinct transcription factor binding sites were identified by DNase1 footprinting studies. These correspond to a cyclic AMP responsive element (CRE), an AP1 element, two core binding factor sites (CBF), and Ikaros.

To determine the relative importance of each factor in the regulation of granzyme B transcription, selected mutations were introduced into each site within the context of the 243 bp promoter. Although none of the individual sites were essential for expression, the combination of the 3' CBF and AP1 binding sites were necessary in activated primary lymphocytes. Their close proximity and requirement in promoter function suggest an important role for protein-protein interactions between AP1 and CBF.

The in vivo importance of this region in transcriptional regulation was demonstrated by in vivo footprinting and DNase1 hypersensitivity analysis in purified $CD8^+$ and $CD4^+$ primary T lymphocytes. One region of highly nuclease accessible DNA was present in activated T cells, which directly correlated with the above mentioned binding sites. This hypersensitive site was not detectable in unstimulated T cells or fibroblasts. Interestingly, all of these transcription factors were found to occupy the endogenous granzyme B promoter in activated, but not resting, T cells. Together, these studies have identified the major regulatory sequences that are responsible for granzyme B transcription in activated T cells and have provided a greater understanding of how cytotoxic T cells are activated.

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LIST OF ABBREVIATIONS

AML	Acute Myeloid Leukemia
AMP	Adenosine Monophosphate
AP1	Activator Protein-1
APC	Antigen Presenting Cell
ATF	Activated Transcription Factor
ATP	Adenosine Triphosphate
bp	Basepairs
C	Constant
cAMP	Cyclic Adenosine 3,5-Monophosphate
CBF	Core Binding Factor
CD	Cluster of Differentiation
cDNA	Complementary DNA
Con A	Concanavalin A
CRE	Cyclic AMP Responsive Element
CTL	Cytotoxic T Cell
D	Diversity
DAG	1,2-Diacylglycerol
DMS	Dimethylsulfate
DNA	Deoxyribonucleic Acid
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediamine tetraacetic acid
EMSA	Electrophoretic Mobility Shift Assay
ERK	Extracellular Signal Regulated Kinase
FACS	Fluorescent Activated Cell Sorting
FITC	Fluoroscein Isothiocyanate
FRK	Fos-Regulating Kinase

GAP	GTPase Activating Protein
G-CSF	Granulocyte Colony Stimulating Factor
GDP	Guanosine Diphosphate
GTF	General Transcription Factors
GTP	Guanosine Triphosphate
HEPES	N-2-hydroxyethylpiperazine-N'-2-Ethanesulfonic Acid
HMG	High Mobility Group
ICAM	Intracellular Adhesion Molecule
IFN	Interferon
IL	Interleukin
IP3	Inositol-1,3,5-Tris Phosphate
ITAM	Immunereceptor Tyrosine Activation Motif
J	Joining
JAK	Janus Kinase
JNK	Jun N-Terminal Kinase
kb	Kilobasepairs
kD	Kilodalton
LFA	Lymphocyte Function-Associated Antigen
LMPCR	Ligation Mediated Polymerase Chain Reaction
MAP	Mitogen Activated Protein
M-CSF	Macrophage Colony Stimulating Factor
MHC	Major Histocompatability Complex
MOPS	3-(N-morpholine)propanesulfonic acid
mRNA	Messenger Ribonucleic Acid
NF-AT	Nuclear Factor of Activated T cells
NK-κB	Nuclear Factor Kappa B
NK Cell	Natural Killer Cell

PAGE	Polyacrylamide Gel Electrophoresis
PARP	Poly(ADP-ribose) Polymerase
PBS	Phosphate Buffered Saline
PCD	Programmed Cell Death
PCR	Polymerase Chain Reaction
PEBP2	Polyoma Enhancer Binding Protein
PIP2	Phosphatidylinositol-4,5-Bisphosphate
PKC	Protein Kinase C
PLC	Phospholipase C
PMA	Phorbol-12-myristate-13-acetate
PMSF	Phenylmethylsulfonyl Fluoride
PTK	Protein Tyrosine Kinase
RLU	Relative Light Units
RNA	Ribonucleic Acid
SDS	Sodium Dodecyl Sulphate
SH2	Src Homology 2 Domain
SH3	Src Homology 3 Domain
STAT	Signal Transducers and Activators of Transcription
SWI/SNF	Switch Deficient/Sucrose Non-Fermentor
TAF	TBP Associated Factor
TBP	TATA Binding Protein
TCR	T Cell Receptor
Th	Helper T Cell
TNF	Tumour Necrosis Factor
TPA	Tetradecanoylphorbol-13-acetate
UV	Ultraviolet
V	Variable

Greek Symbols

α	alpha
β	beta
γ	gamma
δ	delta
ε	epsilon
κ	kappa
η	eta
ζ	zeta

Amino Acid Designations

D	Asp	Aspartic Acid
E	Glu	Glutamic Acid
F	Phe	Phenylalanine
I	Ile	Isoleucine
L	Leu	Leucine
P	Pro	Proline
R	Arg	Arginine
S	Ser	Serine
X		Any Amino Acid
Y	Tyr	Tyrosine

CHAPTER 1

INTRODUCTION

T lymphocytes are the antigen-specific effectors of immune responses. The role of T lymphocytes in the mammalian immune system is to detect virus infected and transformed cells and destroy them before they become a danger to the individual. Upon detection of an anomalous cell, T cells are activated by the recognition of presented peptide antigens. T helper cells (Th) are activated by specialized antigen presenting cells to secrete lymphokines, that recruit and stimulate other lymphocytes. Alternatively, cytotoxic T cells (CTL) are activated by antigens, presented by most cell types, to engage specialized cytolytic machinery to kill the target cell in response to antigenic and lymphokine stimulation.

How T cells become activated in response to antigen and lymphokine stimulation has become the focus of intense research in the past couple of decades. Several detailed accounts of how extracellular information is conveyed into the nucleus have helped to further our understanding of T cell activation. As well, the study of lymphoid-specific gene regulation has identified a multitude of nuclear targets for these cascades.

One approach to understanding the events that occur is to study the specialized subset of genes that are induced during T cell activation. These include the lymphokine and lymphokine receptor genes, as well as the genes that encode components of the killing machinery, such as perforin and granzymes. The studies described in this thesis focus on the regulation of one member of a family of cytotoxic serine proteinase genes, the granzyme B gene. Importantly, the regulation of granzyme B transcription was investigated in newly activated primary murine lymphocytes, which illustrates one of the first attempts to study T cell-specific gene regulation in a physiologically relevant experimental system.

I. T CELLS IN THE IMMUNE SYSTEM

1. DEVELOPMENT OF T CELL SUBSETS

The lymphoid pedigree is derived from pluripotent hematopoietic stem cells located in the marrow of the bone. Mitotic divisions result in the formation of two distinct populations of daughter cells; those that retain pluripotency and those that acquire the capability to differentiate into the myeloid, erythroid, and lymphoid lineages. Although this process of differentiation is still poorly understood, it is of great interest to molecular and developmental biologists seeking an understanding of the molecular basis of differentiation and cell commitment.

T lymphocytes are named as such because they complete their developmental programming in the thymus, a specialized organ in which vast numbers of T cells undergo selection and maturation (reviewed in Abbas *et al.*, 1991; Leclercq and Plum, 1996; Shortman and Wu, 1996; and Weissman, 1994). T cell precursors are believed to commit to the lymphoid lineage before they leave the bone marrow. Once in the blood stream, they migrate to the thymus and 'seed' the organ by a poorly understood homing mechanism. They begin their maturation in the thymic cortex and develop as they migrate into the medulla. As they proceed, they receive sequential educational signals from the thymic epithelium, as well as from other cells in the microenvironment (reviewed in Ritter and Boyd, 1993). The stages of T cell development have mainly been described in terms of the sequential expression of several characteristic T cell surface markers, including the T cell receptor (TCR), and the CD4 and CD8 coreceptor molecules.

Immature thymocytes are devoid of T cell receptors, CD4, and CD8 surface molecules and are sometimes referred to as triple negative cells. One of the first molecular events to occur is the rearrangement of the T cell receptor genes. The TCR genes (α, β, γ, and δ) consist of one or more variable (V), joining (J), and constant (C) region segments. The β and δ genes also contain several diversity (D) region segments. During TCR gene

rearrangement, one each of the V, D, and J coding regions is retained and spliced to the constant region, in the order V-D-J-C. The genetic material intervening these regions is recombined out by specialized recombinases and is permanently deleted. In each T cell, different combinations of V, D, J, and C regions may be selected in order to generate a diverse number of coding sequences. Further diversity may be achieved as nucleotides between the segments are added, deleted, or imperfectly spliced in the recombination process. Rearrangement occurs only in one allele of each TCR gene while the other allele is silenced. This allows each cell a high probability of expressing one unique TCR, while providing a vast repertoire of TCR possibilities (approximately 10^{16}). T cells may express one of two distinct heterodimeric transmembrane TCR complexes: most express an αβ TCR but a smaller population express a γδ TCR. In αβ T cells, the TCRβ gene is rearranged first and its expression precedes the rearrangement of TCRα. The expression of both genes and proteins are essential events in αβ T cell development as mice that have disruptions in either gene have been found to sustain major developmental blocks in thymocyte development (reviewed in Palmer *et al.*, 1993).

The TCR α and β chains are associated with CD3 complex proteins. These proteins are expressed, assembled, and appear on the cell surface concomitant with the αβ TCR. The CD3 complex consists of a number of transmembrane glycoproteins that include the δ, ε, and γ chains in complex with either a homodimer of ζ proteins or a heterodimer of ζ/η proteins. These are stoichiometrically associated with TCRαβ as CD3γδε$_2$/ζ$_2$(orζ/η). The cytoplasmic domains of the CD3 complex function as the main intracellular signaling component of the TCR and will be discussed in more detail in a subsequent section. Just prior to the appearance of the αβ TCR/CD3 complex on the cell surface, the CD4 and CD8 transmembrane glycoproteins are expressed. Thymocytes that express both CD4 and CD8 are referred to as double positive. Functional maturation of double positive cells occurs as these cells begin to express low levels of the TCR/CD3 complex, migrate from the thymic cortex to the medulla, and undergo a rigorous double selection process.

Individual TCRs must be capable of recognizing self-antigens in context with self Major Histocompatibility Complex (MHC) molecules. However, if this interaction results in the activation of the T cell, it is self-reactive and must be eliminated. Of the vast repertoire of TCR specificities, only a subset are capable of recognizing and binding to self MHC molecules. MHC molecules are the antigen associated ligands for the T cell receptor. In the process of positive selection (reviewed by Von Boehmer, 1994), thymocytes with TCRs that are unable to bind to self MHC molecules, and are therefore incapable of triggering an immune response, are induced to die via programmed cell death. Those that do bind are allowed to develop further and are then subjected to the negative selection process.

In negative selection (reviewed by Nossal, 1994), self antigens are presented to the developing T cells, in the context of self MHC molecules, mainly by the cells of the thymic stroma and by specialized antigen presenting dendritic cells (reviewed in Anderson *et al.*, 1996). Those T cells which are activated in response to self antigen are deleted from the repertoire by the induction of programmed cell death. This process ensures that autoreactive T cells are eliminated before they enter the periphery, where they might mount an immune response against self tissues. Together, positive and negative selection eliminate the majority of T cells that enter the thymus and ensure that the few that do emerge are self MHC-restricted and self tolerant.

As $CD4^+/8^+$ cells undergo positive selection, they downregulate the expression of either CD4 or CD8. CD4 and CD8 are the main co-receptor molecules of the TCR complex and play an essential role in T cell activation and function (reviewed in Julius *et al.*, 1993; and O'Rourke and Mescher, 1993). CD4 and CD8 molecules bind to non-polymorphic regions of Class II MHC and Class I MHC, respectively (Doyle and Strominger, 1987; Fleury *et al.*, 1991; Norment *et al.*, 1988; Sanders *et al.*, 1991; Salter *et al.*, 1989). T cells with TCRs that recognize class I MHC, in conjunction with CD8 coreceptors, downregulate the expression of CD4 and develop into mature $CD8^+$ CTLs. Alternatively,

lymphocytes with TCRs that recognize class II MHC, in conjunction with CD4 coreceptors, downregulate the expression of CD8 and become mature $CD4^+$ Th cells. The expression of CD4 and CD8 molecules during thymic development is essential to the maturation of their respective subsets. Mutations that affect either MHC binding or cytoplasmic signaling by the CD4 protein have been found to have profound negative effects on the development of the $CD4^+$ T cell subset. Similarly, the same was found to be true for the CD8 protein in the development of the $CD8^+$ T cell subset (Ramsdell and Fowlkes, 1989; Seong *et al.*, 1992; and reviewed in Miceli and Parnes, 1993).

2. T CELL ACTIVATION

The lymphocytes that do not die in the thymus emerge as either mature $CD4^+$ helper T cells or $CD8^+$ cytotoxic T cells. In the periphery, they are generally quiescent and non-effectual until they receive an appropriate antigenic stimulus. The process of activation induces the cells to proliferate and express the genes, and subsequently the proteins, that allow them to acquire their effector functions, such as cytokine production and cytotoxicity.

a. Antigen Recognition by the TCR

T cells recognize foreign antigens, via their T cell receptor complexes, in the context of membrane-associated Class I and Class II MHC molecules on target cells (Chien and Davis, 1993). Class I MHC molecules are found on essentially all nucleated cells. Class II MHC molecules are present only on antigen presenting cells (APC) such as B cells, macrophages, dendritic cells, endothelial cells, and Langerhans cells. This pattern of MHC expression is important in that Class I-restricted CTL are able to recognize and kill all cells that may become virus infected or transformed, whereas Class II-restricted Th cells may activate distinct immune responses depending on the type of APC it encounters.

Antigens are presented to T cells in the form of short polypeptides, 8-15 amino acids in length, that are non-covalently bound in the antigen presenting groove of MHC

molecules. Peptides associated with Class I MHC are endogenously derived, that is they are generated by the proteolytic degradation of proteins present within the cell (reviewed in York and Rock, 1996). Alternatively, peptides associated with Class II MHC are exogenously derived, or they are the degradation products of extracellular microbes and non-cellular debris that are phagocytosed by the APCs. The variable regions of the TCR α and β glycoprotein chains recognize and bind to two polymorphic α-helical regions of MHC molecules, on either side of the antigenic peptide pocket, in addition to making one or more contacts with the peptide itself (Bjorkman *et al.*, 1987). In effect, the antigen and MHC molecules create a distinct composite surface to which only a select number of TCRs may bind. CD4 or CD8 molecules augment TCR/MHC interaction by binding simultaneously to a non-polymorphic region of the MHC molecule apart from the antigen. The binding of the TCR/CD3 complex, and the CD4 or CD8 coreceptors, to a foreign antigen containing MHC molecule induces several distinct signal transduction pathways that begin at the plasma membrane and terminate in the nucleus.

b. T Cell Receptor Signaling

The αβ chains of the T cell receptor have essentially no intracellular signaling capacity due to their small cytoplasmic domains. The main transmembrane signaling component of the TCR/CD3 complex is derived from the large cytoplasmic domains of the invariant subunits of the CD3 complex (δ, γ, ε, and ζ/ζ or ζ/η) (reviewed in Cantrell, 1996; Crabtree and Clipstone, 1994). These domains contain specialized motifs, termed ITAMs (for immunoglobulin receptor family tyrosine-based activation motifs), that consist of the conserved and strategically spaced amino acids (D/E)XX<u>YXXL</u>/I$(X)_{6-8}$<u>YXXL</u>/I (reviewed in Cambier, 1992; Chan *et al.*, 1994; Samelson and Klausner, 1992; Weiss and Littman, 1994). One ITAM sequence is present in each of the CD3 subunits, except for the ζ chain, which contains three ITAMs. The tyrosine residues (Y) in the ITAMs of the CD3 γ, δ, ε, and ζ chains are rapidly phosphorylated upon antigen binding by the TCR, presumably in

response to receptor aggregation or multimerization. Phosphorylation is mediated by members of the src family of protein tyrosine kinases (PTKs), namely p59fyn, and p56lck. These PTKs are membrane associated by virtue of myristylated glycine residues in their N-terminal domains. p56lck interacts with the cytoplasmic domains of the CD4 and CD8 co-receptors while p59fyn is associated with the CD3 chains. Coengagement of the TCR/CD3 complex and CD4 or CD8 molecules localizes p56lck and p59fyn to the internal TCR/CD3 signaling complex (Figure 1-1).

Activation of the src kinases has been proposed to precede the phosphorylation of the ITAMs. The phosphorylated ITAMs then serve as a docking site for a 70 kD, non-myristylated cytoplasmic protein termed ZAP-70 (Chan *et al.*, 1991; Chan *et al.*, 1992; Iwashima *et al.*, 1994). ZAP-70 is activated by tyrosine phosphorylation by an as yet undesignated kinase. It associates with the ITAMs via its two src homology 2 (SH2) domains which are strategically spaced such that each one directly interacts with one of the two phosphorylated tyrosine residues of the ITAMs. The function of ZAP-70 following phosphorylation and association with the ITAMs is unclear but it may function as a kinase that phosphorylates subsequent substrates in the signal transduction cascade.

Several proteins are known to be phosphorylated in response to T cell receptor engagement. These include various plasma membrane proteins, kinases, and numerous downstream enzymes. The phosphorylated membrane proteins include the CD3 δ, ε, γ, and ζ chains and other receptor molecules, such as CD5 and CD6. The src kinases (lck and fyn) and the syk kinases (ZAP-70 and syk) are themselves phosphorylated, in addition to the downstream kinase, MAP-kinase. Other PTK substrates include several protooncogene proteins, such as Vav, c-cbl, and shc, the cytoskeletal protein Ezrin, phospholipase Cγ1, GTPase-activating proteins (GAPs), and the valsolin containing protein (reviewed in Cantrell, 1996; Weiss, 1994). Although these proteins are known targets of the TCR regulated kinases, the functional significance of many of the phosphorylations are not known.

Figure 1-1 The T Cell Receptor and Cytokine Receptors Activate Several Signaling Transduction Pathways.

Target cell binding through MHC/TCR/CD8 interactions elicit a variety of intracellular signaling events from the cytoplasmic domains of the CD3 complex. These events include the activation of protein-tyrosine kinases (PTK) and protein kinase C (PKC) pathways, plus the increase in intracellular calcium concentrations. These pathways trigger the activation of a number of transcription factors, by a variety of mechanisms. The JAK/STAT pathway of cytokine signal transduction and the protein kinase A (PKA) pathway are shown as well.

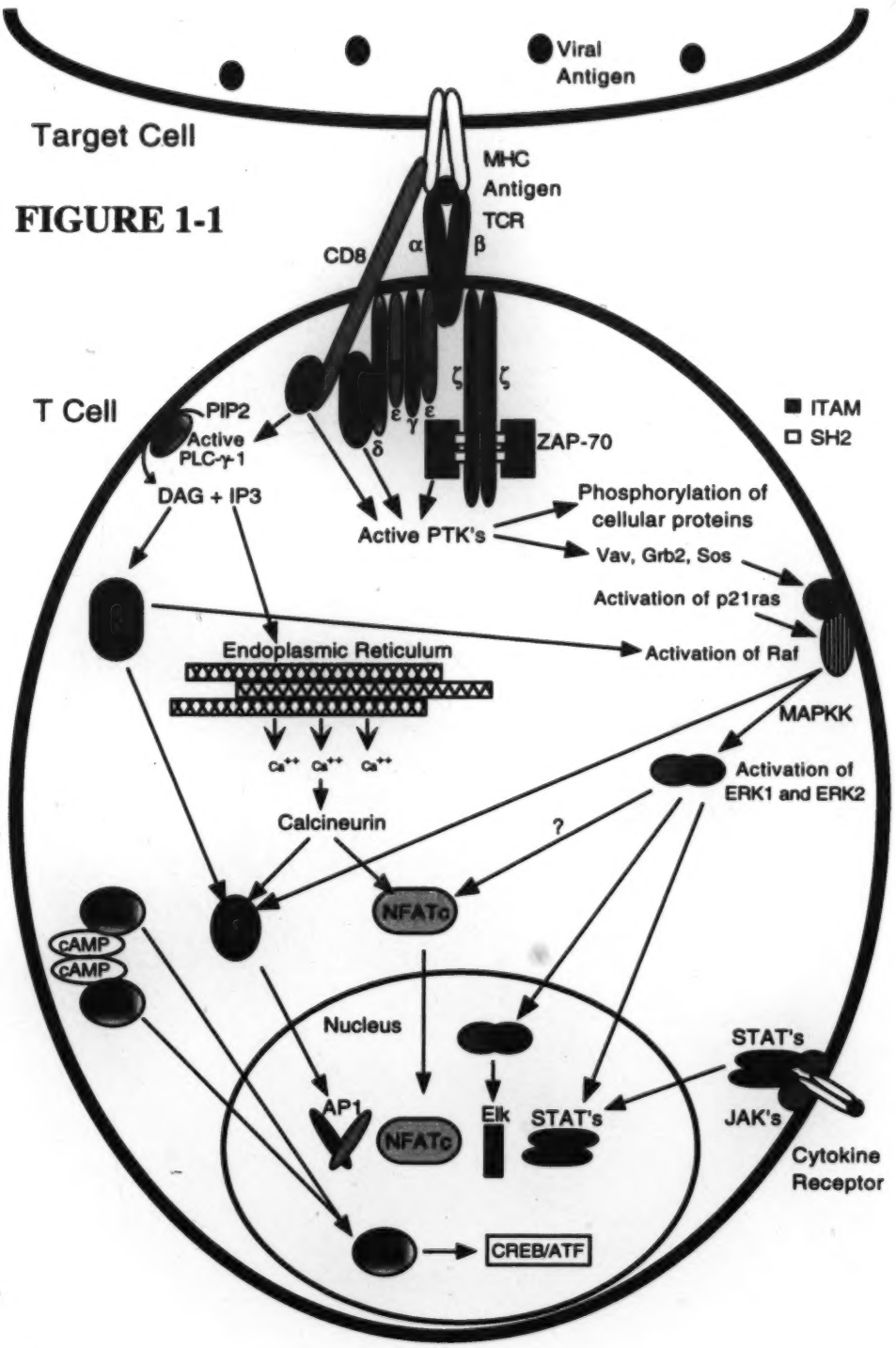
Viral Antigen
Target Cell
MHC Antigen
TCR
CD8
α
β
T Cell
PIP2
Active PLC-γ-1
DAG + IP3
ε γ ε
ζ ζ
δ
ZAP-70
ITAM
SH2
Active PTK's
Phosphorylation of cellular proteins
Vav, Grb2, Sos
Activation of p21ras
Activation of Raf
Endoplasmic Reticulum
MAPKK
Ca++ Ca++ Ca++
Activation of ERK1 and ERK2
Calcineurin
?
NFATc
cAMP
cAMP
Nucleus
STAT's
AP1
NFATc
Elk
STAT's
JAK's
Cytokine Receptor
CREB/ATF

FIGURE 1-1

c. Regulation of Phosphatidylinositol Metabolism

One of the best characterized T cell signal transduction pathways involves the metabolism of the membrane phospholipid phosphatidylinositol, which is hydrolyzed by phospholipase Cγl (PLCγl). PLCγl is activated by tyrosine phosphorylation in response to TCR stimulation, most likely by p56lck (Weiss *et al.*, 1991). It was shown to be recruited to the plasma membrane, via its SH2 domains, by a 36 kD membrane protein that is also tyrosine phosphorylated in response to TCR engagement (Sieh *et al.*, 1994). PLCγl catalyses the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) into the second messengers inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). IP3 diffuses into the cytoplasm where it interacts with IP3-gated calcium transport channels of the endoplasmic reticulum. An induced conformational change in the channels triggers the release of intracellular calcium stores into the cytoplasm. DAG remains membrane-associated and activates several of the protein kinase C (PKC) isozymes and increases their affinity for calcium, also an activator of PKCs.

It is important to note that the experimental use of phorbol esters and calcium ionophores to activate T cells in vitro mimics this signal transduction pathway. The phorbol ester 12-*O*-Tetradecanoylphorbol-13-acetate (TPA) structurally resembles DAG and is a potent activator of PKC. Interestingly, the use of either phorbol esters or calcium ionophores alone is not capable of inducing T cell activation. This implies that two distinct types of signals are necessary for full T cell activation; one calcium-regulated and the other kinase-regulated.

d. p21ras Activation and MAP Kinases

A consequence of TCR-mediated PTK stimulation, and possibly PKC stimulation, is the activation of the p21ras guanine nucleotide binding proteins (reviewed in Cantrell, 1996; Downward *et al.*, 1992). p21ras proteins are associated with the inner cytoplasmic membrane via covalently attached polyisoprenoid groups near their carboxy termini. The

GTP bound form of p21ras is active; however, a weak GTPase activity makes p21ras function dependent upon interaction with guanine nucleotide exchange proteins and the presence of cellular GTP. Guanine nucleotide exchange proteins facilitate the reciprocation of GDP for GTP and include the proteins Vav, Sos, and C3G. Alternatively, the GTPase activating proteins (GAPs) promote the hydrolysis of GTP by p21ras, thereby inhibiting its activity. This repression is overcome upon TCR stimulation by the PKC-dependent phosphorylation of GAP, which inhibits its GTPase activating effects (reviewed in Polakis and McCormic, 1993; Margolis *et al.*, 1992).

Vav is generally restricted to hematopoietic cells and is tyrosine phosphorylated in response to T cell activation by p56lck. The phosphorylated form of Vav has increased guanine nucleotide releasing activity for p21ras in vitro (Gulbins *et al.*, 1993). The Sos guanine nucleotide exchange factor is associated with Grb2, a 26 kD adaptor protein which contains one SH2 domain and two SH3 domains (reviewed in McCormick, 1993). Grb2 binds to Sos via its SH3 domains and links Sos to the plasma membrane by binding to tyrosine phosphorylated proteins through its SH2 domain. Two different proteins have been found to interact with Grb2. One molecule is a 36 kD membrane protein that becomes phosphorylated upon TCR ligation (Bunday *et al.*, 1994; Sieh *et al.*, 1994). The formation of p36/Grb2/Sos complexes occurs rapidly following activation and their presence correlates with p21ras activity. The other protein is the adaptor protein Shc (Osman *et al.*, 1995; Ravichandran *et al.*, 1993). Shc is tyrosine phosphorylated in response to T cell activation and the Shc/Grb2/Sos complex may also be involved in p21ras regulation. A different complex that was found to form in response to T cell activation involves the C3G guanine nucleotide exchange protein. C3G interacts with the three SH3 domains of the Crk adaptor protein, while the two SH2 domains of Crk interact with tyrosine phosphoproteins, in a manner analogous to Grb2 (Sawasdikosol, 1995). Unfortunately, the exact function of Grb2 and C3G containing complexes in p21ras regulation in T cells is not yet known.

The downstream target of activated p21ras has recently been identified as the serine/threonine kinase Raf-1. Direct protein-protein interactions between p21ras and Raf-1 have been shown to occur but the mechanism of Raf-1 activation is still unknown (Crews and Erikson, 1993). Activated Raf-1 phosphorylates and activates mitogen-activated protein (MAP) kinase-kinase, which in turn phosphorylates and activates the MAP kinases ERK1 and ERK2 (reviewed in Cooper, 1994; Marshall, 1994).

3. CONSEQUENCES OF SIGNAL TRANSDUCTION IN T CELLS

The few linear signaling pathways that have been characterized were the result of many concerted efforts to identify the molecular events that lead to the expression of cytokine genes in T cells, particularly the IL-2 gene. Many of these pathways contain integrated or interdependent branches of the p21ras/MAP kinase pathway, the calcium/PKC pathway, and the JAK/STAT pathway. The ultimate targets for these transducing events are nuclear transcription factors, which may be activated by a variety of different mechanisms and often by multiple pathways (see Table 1-1 and Figure 1-1).

a. p21ras/MAP Kinase-Mediated Signals

The 'extracellular signal regulated kinases' ERK1 and ERK2 become phosphorylated upon TCR stimulation via the p21ras/Raf/MAP kinase pathway. ERK 1 and ERK2 have been shown to enter the nucleus and phosphorylate the transcription factors Elk-1, SAP-1, and AML-1 (also known as core binding factor) (reviewed in Hill and Teisman, 1995; Treisman, 1994). Phosphorylation on a cluster of serine/threonine motifs in the carboxy-termini of Elk-1 and SAP-1 is believed to increase their DNA binding activity to the serum response element, which is present in many activation-responsive genes including the c-fos promoter. The phosphorylation of AML1 on two specific serine residues potentiates its transcriptional transactivation capacity when it is bound to its recognition sequence in an

Table 1-1: Properties of Transcription Factors That are Targets for Signaling Pathways

(adapted from Hill and Treisman, 1995)

Transcription Factor Family	DNA-Binding Domain	DNA-Binding Properties and Recognition Sites	Properties Regulated by Signal	Activating Signal
Elk-1, SAP-1	Ets domain	Binds SREs as ternary complex with SRF	Transactivation, DNA binding	TCR engagement via p21ras/ERK pathway
CREB/ATF	bZIP	Binds CRE as homodimer or as heterodimer with other ATF family members	Transactivation; association with coactivator CBP	PKA pathway
AP1 (Jun)	bZIP	Binds AP1 elements as homodimers or with Fos proteins	DNA binding; transactivation; association with coactivator CBP?	UV irradiation, PKC activation, cytokines
STAT	Novel	Bind as homo- or heterodimers to GAS elements and related sites. STATs 1 and 2 bind as part of heterotrimeric complex (ISGF3) to ISREs	Dimerization; Nuclear localization	Cytokines and growth factors
NFκB	Rel homology domain	Binds κB sites as homo- or heterodimers with other family members	Nuclear localization; release from the inhibitor, IκB	Lymphokines, TNFα, TCR engagement, PKC activation
NF-AT complex (NF-ATc, AP1)	Rel-like (NF-ATc) bZIP (AP1)	NF-AT sites: composite element containing NF-ATc and AP1 recognition motifs	Nuclear localization (NF-ATc); synthesis/transactivation (AP1)	TCR activation

enhancer (Tanaka *et al.*, 1996).

Other targets for the p21ras/Raf/MAP kinase pathway include the Jun kinase JNK and the Fos kinase FRK. Jun and Fos are ubiquitously expressed transcription factors that bind to AP1 elements in many activation-responsive promoters. Phosphorylated JNK has been found to associate with the N-terminus of c-Jun. JNK activates the transcriptional transactivation domain of Jun by phosphorylating important regulatory serine/threonine residues within it (Binetruy *et al.*, 1991; Pulverer *et al.*, 1991). Similarly, phosphorylated FRK activates Fos by phosphorylating a threonine residue in its transcriptional transactivation domain (Deng and Karin, 1994).

b. Calcium and PKC-Mediated Signals

T lymphocyte activation results in a biphasic increase in intracellular calcium. Initially, calcium is released from intracellular stores in the endoplasmic reticulum by IP3 action. This results in a transient peak in Ca^{2+} concentration followed by a lower sustained plateau phase that is mediated by Ca^{2+} entry into the T cell from extracellular sources. Calcium chelating agents or agents that inhibit calcium release have a dramatic inhibitory effect on T cell activation and proliferation. This is mainly due to the calcium binding proteins that play an integral role in T cell signal transduction. One of the better understood calcium-regulated signal transduction pathways is mediated by the serine/threonine phosphatase, calcineurin (Clipstone and Crabtree, 1992). The calcium bound form of calcineurin is believed to regulate the translocation of the cytosolic component of the transcription factor NF-AT (nuclear factor of activated T cells) into the nucleus. NF-ATc is a preexisting phosphoprotein that is anchored in the cytoplasm by an unknown mechanism. Upon T cell induction, calcineurin is presumed to dephosphorylate either NF-ATc or its cytoplasmic anchor, thereby allowing it to translocate into the nucleus (Jain *et al.*, 1993).

The activation of the serine/threonine protein kinase C family has many diverse consequences in T cell activation (reviewed in Buchner, 1995; Szamel and Resch, 1995).

Isoforms of PKC proteins have been found to reside in both the cytoplasm and the nucleus. As well, other isoforms have been found to translocate into the nucleus in response to certain stimuli, such as DAG or phorbol esters. PKC is believed to play a key role in the activation of transcription as many of its substrates are transcription factors or integral nuclear proteins. One transcription factor that is indirectly activated by PKC is NFκB. The cytoplasmic anchor for NFκB, IκB, is inactivated by PKC phosphorylation and is subsequently degraded. This releases NFκB from its anchor and unmasks the nuclear localization sequence that allows it to translocate into the nucleus (reviewed in Baldwin, 1996). Interestingly, PKC may support the regulation of chromatin structure or DNA replication and repair. Lamin B and DNA topoisomerase I have been shown to be substrates for PKC both in vitro and in vivo. In vitro, PKC has been found to phosphorylate lamins A and C, histones H1, H2B, and H4, RNA Polymerase II, and DNA polymerase α/β, but the physiological relevance of these in vitro findings has yet to be determined. Interestingly, the cytoplasmic PKCα protein was found to phosphorylate Raf-1 both in vitro and in vivo, thus activating the MAP kinase pathway and providing a link between phosphatidylinositol metabolism and the p21ras pathway (Sozeri *et al.*, 1992; Kolch *et al.*, 1993).

Activation of the Jun transcription factor requires costimulation by calcium and PKC-dependent pathways and involves two distinct mechanisms, one involving phosphorylation and the other involving dephosphorylation. The phosphorylation of Jun proteins by JNK was found to require both calcium and PKC-mediated signals (Su *et al.*, 1994). Individual signals mediated by either calcium ionophores or phorbol esters alone did not result in the activation of JNK activity or the subsequent activation of Jun proteins. Phosphorylation of Jun by JNK results in the activation of its transcriptional transactivation domain. Alternatively, PKC proteins were determined to indirectly regulate the DNA binding activity of Jun by dephosphorylation (Boyle *et al.*, 1991). The Jun protein in resting cells does not bind DNA due to the presence of phosphorylated serine and threonine residues in

a region just upstream of its DNA binding domain. These residues are dephosphorylated in response to PKC activation, presumably by a PKC-dependent phosphatase, and the DNA binding capacity of Jun is activated. Confirmation of the signaling requirements for AP1 activity was provided by Rincon and Flavell (1994) in primary lymphocytes. Transgenic mice were created that possessed a reporter gene under the control of multimerized AP1 binding sites inserted into their genomes. The transcriptional activation potential of AP1 required both calcium and PKC-mediated signals (necessary for JNK activity) while the DNA binding activity required only activated PKC derived signals (Jun phosphatase activity).

An interesting way in which the integration of multiple signal transduction pathways elicits a nuclear response is exemplified by the NF-AT transcription factor complex. The NF-AT complex consists of the cytoplasmic calcium-dependent component, NF-ATc and a nuclear component consisting of the Fos and Jun proteins. As mentioned above, the Fos and Jun proteins are activated by PKC and p21ras-dependent mechanisms. Upon T cell induction, the increase in intracellular calcium concentration allows the calcineurin-regulated NF-ATc component to translocate into the nucleus where it forms a heteromeric complex with the activated forms of the Fos and Jun proteins.

4. OTHER PATHWAYS INVOLVED IN T CELL ACTIVATION

a. JAKs and STATs

Cytokines and growth factors also play key roles in T cell activation. When cytokines bind to their cognate surface receptors, receptor cytoplasmic domains transduce a variety of molecular signals that may either augment activation or be an essential component of it. Hematopoietic cytokine receptors lack intrinsic tyrosine kinase activity but one method of molecular signaling that has been found to be associated with several cytokine receptors is the JAK/STAT signal transduction pathway. Janus kinases (JAKs) are a family of tyrosine kinases composed of JAKs 1, 2, and 3, and Tyk2 in mammalian cells

(reviewed in Hill and Treisman, 1995; Kishimoto *et al.*, 1994; Winston and Hunter, 1996). The binding of a cytokine to its receptor results in the dimerization, or multimerization, of the receptor subunits. This leads to the association of one or more JAKs with the multimerized cytoplasmic receptor domains. Whether there is a direct association of JAK proteins with the receptors is not known, however, JAK activation was found to be dependent on the integrity of a proline-rich sequence present in the cytoplasmic domains of the receptor subunits. Although JAKs 1 and 2 are the most commonly activated kinases, between one and three JAKs may associate and become activated, depending on the receptor.

JAK kinases phosphorylate certain tyrosine residues on the cytokine receptor cytoplasmic domains. These phosphotyrosines serve as binding sites for the SH2 domains of members of the STAT (signal transducers and activators of transcription) family of proteins. Upon association with the receptor and their associated JAKs, STATs are phosphorylated by JAKs on conserved tyrosine residues in their C-termini. There are six known mammalian STAT proteins (1 through 6) and between one and three STATs may be activated by a given receptor. The specificity of JAKs for the different STATs is complex. Although different receptors may activate similar JAKs, the STATs that become activated may be quite diverse. For example, JAK1 and JAK3 are activated by both the interleukin 2 receptor and the interleukin 4 receptors but the IL-2R activates STAT3 and STAT5 while the IL-4R activates STAT6 (Beadling *et al.*, 1994; reviewed in Hill and Treisman, 1995; Rebollo *et al.*, 1996). The specificity of STAT activation may be determined by differential affinities of their SH2 domains for the different modular tyrosine based motifs of the phosphorylated receptor domains (Stahl *et al.*, 1995). Recent evidence suggests that serine phosphorylation of STATs is also required for function. Serine phosphorylation is likely mediated by MAP kinases, which appear to be alternatively regulated by JAKs in addition to p21ras (David *et al.*, 1995; Wen *et al.*, 1995; Zhang *et al.*, 1995; and reviewed in Winston and Hunter, 1996).

The phosphorylation of STAT proteins allows them to dimerize via reciprocal SH2 domain interactions and dissociate from the receptor. STAT dimers migrate into the nucleus where they bind to specific DNA response elements, such as the Sis-inducible element in the fos promoter, and function as activators of transcription.

b. cAMP and Protein Kinase A

Cyclic AMP is a ubiquitous regulator of cellular activation and plays a definitive role in T cell stimulation (reviewed in Karin and Smeal, 1992; Sassone-Corsi, 1995). The binding of extracellular ligands to G protein-associated receptors, like the TCR, results in the activation of the membrane bound enzyme adenylate cyclase, which in turn generates an increase in intracellular cAMP. cAMP binds to two sites on the regulatory subunit of protein kinase A (PKA) and releases it from its catalytic subunit. The catalytic subunit is then translocated into the nucleus where it may phosphorylate a variety of cAMP-responsive transcription factors, primarily on serine residues in the context of X-R-R-X-S-X motifs. The ubiquitously expressed family of transcription factors, termed CREB/ATF, bind to cAMP-responsive elements in a wide variety of genes and include members that are directly phosphorylated by activated PKA. CREB and CREM are such proteins that are phosphorylated on conserved serine residues in their specialized P-box domains. These phosphorylated motifs allow the transcription factors to interact with their coactivator protein (CBP) and transactivate transcription.

5. EFFECTOR FUNCTIONS OF T CELLS

Naive lymphocytes, those that have not yet been exposed to an activating antigenic stimulus, are small and generally quiescent. In addition, they do not posses any of the effector functions that are characteristic of activated T cells, such as cytokine production or cytotoxicity. Antigen specific activation results in the proliferation and expansion of the population and in the de novo production of the hundreds of proteins that allow T cells to acquire their functional potentials.

a. Cytokine Production by Helper T Lymphocytes

The function of helper T cells (Th) in the immune system is to detect infections and produce molecular regulators of the immune response that would best deal with the invading pathogen. Th cells are activated by making cell-cell contacts with antigen presenting cells through TCR/CD4-mediated interactions with antigen/Class II MHC complexes, and through various accessory molecules. Th cell activation results in the transcription and production of a variety of small bioactive peptides called cytokines. These proteins diffuse into the surrounding microenvironment, recruit other lymphocytes to the infection site, and activate their responses.

The specificity of an immune response, either humoral or cellular, to a particular infection is dependent on the type of cytokines produced by the Th cell (reviewed in Abbas *et al.*, 1996; Paul and Seder, 1994; Seder and Paul, 1994). Different patterns of cytokines are secreted by the two major subsets of Th lymphocytes, Th1 and Th2. Humoral immunity is primarily mediated by the Th2 subset whereas cellular immunity is mediated by the Th1 subset. For example, the cytokines IL-4, IL-13, and interferon-γ induce humor immunity by stimulating B cells to produce antibodies to clear soluble antigens. Alternatively, IL-2 and interferon-γ induce cellular immunity by stimulating cytotoxic T cells to kill virus infected or transformed cells.

b. Cytotoxic T Lymphocytes

Cytotoxic T cells (CTL) constitute the $CD8^+$ branch of lymphocytes in the immune system. CTLs selectively destroy virus infected and transformed cells within the body before the virus or tumor can spread. CTLs are activated by making cell-cell contacts with antigen/Class I MHC expressing cells through TCR/CD8-mediated interactions. Activation results in the transcription and production of the constituents of the cytolytic machinery. Cytotoxicity by CTLs can be accounted for by two distinct mechanisms (reviewed in Atkinson and Bleackley, 1995; Berke, 1994; Berke, 1995; Kägi *et al.*, 1996). One, the fas pathway, involves receptor/ligand-mediated induction of apoptosis in the target cell. The other involves the directional exocytosis of lytic granules into the intercellular junction between the T cell and the target cell and the induction of target cell apoptosis by the granule proteins. In both mechanisms the T cell delivers its 'lethal hit', detaches from the target cell, and recycles to kill remaining targets.

i) Granule-Mediated Killing

The granule exocytosis model of cytotoxicity has been fairly well characterized in recent years. Many concerted efforts have provided various step by step accounts, both morphological and molecular, of the lytic mechanism from the point of cell-cell contact to the induction of apoptosis in the target. The first event to occur is the formation of a cell-cell conjugate between an activated CTL and its target. The contact site covers a relatively large surface area of both cells and extensive interdigitation of the plasma membranes is observable by electron microscopy (Berke, 1993). Adhesion is mediated by interactions between surface molecules on both cells. These include TCR/CD3/CD8 - MHC interactions as well as interactions between CTL coreceptor molecules such as CD2, CD28, and LFA-1 and their corresponding target cell ligands LFA-3, B7, and ICAM-1 (Azuma *et al.*, 1992; Hibbs *et al.*, 1991). Following the establishment of contact, microtubule organizing centers and centrioles are believed to direct the active realignment of lytic

granules towards the side of the CTL that faces the target cell (Bykovskaja *et al.*, 1978; Geiger *et al.*, 1982; Yanelli *et al.*, 1986).

To deliver the lethal hit, lytic granules migrate towards the point of target cell contact along microtubules in a kinesin-dependent manner (Burkhardt *et al.*, 1993). Granule membranes fuse with the outer cellular membrane and the granule proteins are spilled out into the intercellular junction (Henkart and Henkart, 1982; Jenne and Tschopp, 1988; Takayama and Sitovsky, 1987). The specific directionality of the hit ensures that only the target cell is destroyed and that lytic molecules do not damage neighboring cells. Granule-mediated exocytosis is dependent on the presence of extracellular calcium and can be inhibited with calcium chelating agents or with drugs that block the secretory pathway, like cytochalasin B or monensin.

ii) Granules

Lytic granules have been implicated as the central component of the cytolytic machinery as isolated granules are able to nonspecifically lyse a variety of cell types (Henkart *et al.*, 1984). Granules appear in the cytoplasm soon after activation and accumulate in large quantities in CTL and are constitutively present in natural killer (NK) cells. They resemble lysosomes in morphology in that they are acidic (pH 5.5) and they contain several lysosomal proteins including arylsulphatase, glucuronidase, hexosamidase, cathepsin D, lamp-1, lamp-2, and CD63 (Peters *et al.*, 1991; Tschopp and Nabholz, 1990). The main lytic components of granules are the pore forming protein perforin (reviewed in Liu *et al.*, 1995) and a number of serine esterases termed granzymes (reviewed in Atkinson and Bleackley, 1995; Smyth and Trapani, 1995). An important feature of granules is that they contain negatively charged chondroitin sulphate proteoglycans, which have been found to form protective complexes with the highly basic granzymes and perforin both inside the granules and immediately after exocytosis. The proteoglycans are resistant to granzyme cleavage in the acidic environment of the granules

and are believed to protect granule proteins from granzyme induced proteolysis (Masson *et al.*, 1990; Stevens *et al.*, 1989). Other resident proteins of the granules include the granzyme processing protein dipeptidyl peptidase, the 15 kD RNA binding protein TIA-1, the calcium and perforin binding protein calreticulin, the tumour necrosis factor-like protein leukalexin, and leukophysin, a protein implicated in granule mobility. Several of these proteins may have important functions in the killing mechanism but their exact functions have yet to be determined.

iii) Granzymes and Perforin

Granzymes are a highly conserved family of CTL and NK cell-restricted serine proteinases that make up approximately 90% of total granule protein (reviewed in Smyth and Trapani, 1995). To date, seven granzymes (A-H) have been identified in mice (see Table 1-2) and three have been discovered in humans (A, B, and CCPX). Although they are all rich in basic amino acids and are highly conserved at the amino acid level, their substrate specificities vary. Granzyme A exhibits trypsin-like activity and cleaves substrates preferentially after arginine residues, whereas granzyme B cleaves preferentially after aspartic acid residues. Granzyme C cleaves after asparagine residues and granzymes D, E, F, G, and H cleave at phenylalanine residues. Granzyme proteins are packaged into granules as inactive precursors, but are activated shortly thereafter by the removal of an inhibitory N-terminal dipeptide by dipeptidyl peptidase (also known as cathepsin C) (McGuire *et al.*, 1993). Granzymes A and B are known to contain mannose-6-phosphate glycosylated residues. Targeting of the granzymes to the lytic granules (except for the unglycosylated granzyme C) is achieved by the mannose-6-phosphate receptor, which is abundant in both granules and lysosomes (Griffiths and Isaaz, 1993; Jenne *et al.*, 1988). Granzymes have been implicated in granule-mediated cytotoxicity by experiments showing that serine protease inhibitors blocked, or severely inhibited, the cytolytic activity of CTLs

Granzyme	Mass in kD	Substrate Specificity
Granzyme A	60	Arg/Lys
Granzyme B	29-33	Asp/Glu
Granzyme C	27	Asn/Ser
Granzyme D	35-50	Phe/Leu
Granzyme E	35-50	Phe/Leu
Granzyme F	35-45	Phe/Leu
Granzyme G	33	Phe/Leu

Table 1-2. A list of the known murine granzyme proteins, their relative molecular weights, and their preferred substrate specificities. Adapted from Atkinson and Bleackley, 1995 and Smyth and Trapani, 1995.

(Hudig *et al.*, 1981; Hudig *et al.*, 1991). Although, it is unknown whether these inhibitors also blocked other endogenous proteases that may be equally important in the killing mechanism.

Perforin is a 65-70 kD granule protein (reviewed in Liu *et al.*, 1995; Podack *et al.*, 1991) that shares homology with the channel forming complement proteins of the immune system. Perforin is named as such because it perforates target cell membranes by forming barrel-like polymeric transmembrane channels which have been visualized by electron

microscopy (Dourmashkin *et al.*, 1980). In T cells, perforin is post-translationally modified by N-glycosylation and by the cleavage of a 20 residue leader peptide in the Golgi network. Unlike granzymes, it is targeted to granules through the golgi network independently of the mannose-6-phosphate receptor (Burkhardt *et al.*, 1989). In granules, perforin monomers are complexed with proteoglycans and calreticulin. Following exocytosis, the change in extracellular pH, and possibly salt concentration, is believed to release perforin from these inhibitory complexes. Perforin has been observed to bind to biomembranes in a calcium-dependent process that appears to involve phosphorylcholine moieties as membrane receptors (Tschopp *et al.*, 1989). The monomers then insert into the membrane and polymerize to form tubular aggregates of variable diameters. These perforin channels have been observed to range from 5-20 nm in diameter, depending on the number of monomers that form each channel (Masson and Tschopp, 1985). Importantly, these channels appear to allow the bi-directional flow of fluids, as well as relatively large proteins, across cell membranes. This property of perforin channels led many researchers to believe that perforin was the only essential lytic molecule of granules, as it was observed that perforin alone could cause severe damage to cells by osmotic lysis (Duke *et al.*, 1989) and perforin knockout mice displayed severely inhibited granule-mediated cytotoxicity (Kägi *et al.*, 1994). However, membrane damage alone could not account for all of the events that subsequently occurred in target cells upon CTL attack.

iv) Uptake of Granule Components and Killing

Purified granzyme proteins, when incubated with cells, require perforin for uptake and cytotoxicity (Shi *et al.*, 1992a). While it is generally accepted that perforin forms pores in the plasma membrane of the target cell, the mode of entry of the granzymes and other granule proteins into the target is less clear. Two potential models of how granule components gain access to the target cell have been proposed. One model proposes that granule proteins passively diffuse into the target through the perforin channels. The other

model involves the facilitated uptake of granule components by endocytosis, depicting an effort by the target cell to repair the membrane damage created by the perforin lesions. The latter mechanism is currently favored as it was found that reagents that block endocytosis also inhibit CTL-mediated cytotoxicity (Shi *et al.*, 1992b).

One of the first events to occur after the uptake of granule proteins is the intranucleosomal fragmentation of the target cell DNA into oligonucleosome sized units. This is followed by condensation of the nuclear chromatin, cell shrinkage, blebbing of the membrane, and the disintegration of the cell into numerous membrane sealed fragments. These apoptotic bodies as they are referred to, are efficiently removed by phagocytosis, thereby preventing an inflammatory response to the dead tissue. These morphological features of cytotoxicity are very similar to programmed cell death (PCD), or apoptosis. PCD occurs in most multicellular organisms as a systematic mechanism of developmental and homeostatic cell elimination (i.e. the positive and negative selection of thymocytes).

Evidence of granule protein involvement in target cell killing had been indirect until relatively recently. While the presence of perforin and granzymes has been correlated with cytotoxicity, no absolute link between either protein and target cell death was apparent. The generation of granzyme B knockout mice indicated a role for this protein in DNA fragmentation as CTL from granzyme B null mice showed an impaired ability to induce rapid DNA fragmentation in target cells (Heusel *et al.*, 1994). Furthermore, immunohistochemical studies of target cells that were incubated with purified granzyme B protein have shown granzyme B to localize to the nucleus and far-Western blotting detected the association of granzyme B with an 80 kD nuclear protein (Pinkoski, *et al.*, 1996). Recently, a cytoplasmic target cell substrate for granzyme B was identified in the Bleackley laboratory and is believed to be directly involved in DNA fragmentation. An interleukin-β1 converting enzyme-like protein called CPP32 had been demonstrated to be proteolytically activated by cleavage at an Asp residue at an early step in PCD (Nicholson *et al.*, 1995; Tewari *et al.*, 1995). Activated CPP32 subsequently cleaves poly(ADP-ribose) polymerase

(PARP), an enzyme involved in DNA repair. PARP inactivation correlates with the onset of PCD and the loss of a DNA repair enzyme is believed to accelerate the DNA fragmentation process. Granzyme B was found to cleave, and thereby activate, CPP32 both in vitro and in vivo with its rare Asp-ase activity (Darmon *et al.*, 1995). Thus, a direct link between killer cell molecules and the pre-programmed death machinery of target cells had been established. Although CPP32 was the first identified physiological substrate for a granzyme protein, the search for more granzyme substrates will likely continue for some time. Moreover, it is anticipated that these substrates will be just as interesting and revealing as the first.

II. TRANSCRIPTION

The transcriptional induction of specific genes reshapes cellular morphology and function in response to environmental stimuli. Perforin, granzymes, and other components of the killing machinery are produced de novo upon antigenic stimulation of cytotoxic T cells. This is achieved by the induction of multiple signal transduction pathways whose endpoints intersect in the nucleus and result in the transcription of CTL-specific genes.

Transcriptional initiation by eukaryotic RNA polymerase depends upon a combination of specific DNA sequence elements. These elements are bipartite in structure and include the core promoter sequences and their regulatory sequences, such as enhancers or silencers. The core promoter determines the transcription start site and directs the assembly of the preinitiation complex, which consists of the core proteins of the basal transcription apparatus. Regulatory elements are highly variable, gene specific binding sites for a number of ubiquitous as well as tissue-specific transcription factors. Regulatory elements may be positioned near the promoter, or at distal sequences upstream or downstream of the gene.

Three general classes of eukaryotic RNA polymerases exist and they control the transcription of different types of genes. RNA polymerase I is located in the nucleolus and directs the synthesis of ribosomal RNA. RNA polymerase II transcribes protein encoding messenger RNAs and several small nuclear RNAs. RNA polymerase III transcribes transfer RNAs and several other small RNAs. Each of these polymerases recognize unique core promoter structures inherent to the different gene classes and each associates with different set of auxiliary proteins.

1. THE BASAL TRANSCRIPTION APPARATUS

RNA Polymerase II (Pol II) is the core enzyme responsible for the transcription of nuclear genes encoding messenger RNAs. On its own, it cannot recognize or bind to core promoter sequences. Therefore, a universal set of auxiliary proteins exist, known as general transcription factors (or GTFs), that associate with RNA Pol II and enables it to bind to promoters and initiate transcription. The combination of core Pol II subunits, many GTFs, plus several other proteins required for transcriptional initiation, constitute the large multisubunit complex called the RNA Polymerase II holoenzyme. The RNA Pol II holoenzyme transcribes most, if not all, protein encoding class II genes and may include accessory functions such as DNA repair.

The basal transcription apparatus consists of RNA Pol II, TFIID, which contains the TATA binding protein (TBP) and its associated factors (TAFs), TFIIB, TFIIE, TFIIF, and TFIIH (see Table 1-3). All of these proteins are highly evolutionarily conserved. In recent years, most of the cDNAs encoding these proteins have been isolated from yeast, *Drosophila*, rodents, and humans. These cDNAs have provided researchers with the necessary tools in which to study these proteins structure and function, as well as perform mutational studies. Together, these investigations have provided many valuable insights into GTF functions and interactions within the preinitiation complex, as well as providing information regarding the interactions between these factors and transcriptional activators.

a. The Preinitiation Complex

The transcriptional preinitiation complex is assembled in a stepwise fashion on class II promoters (reviewed in Roeder, 1996). The first event to occur is the binding of the TBP/TAF complex to an A/T rich sequence element that is present in many genes and is referred to as the TATA box. TBP binds in a sequence specific manner, unlike the other GTFs, in the minor groove of DNA and induces a 90° bend in the DNA molecule (reviewed in Burley and Roeder, 1996). The second factor to bind is TFIIA, which binds upstream of TBP and stabilizes TBP/DNA interactions. TFIIB then binds and further stabilizes the complex by making several contacts with nucleotides both upstream and downstream relative to TBP on the opposite face of the DNA helix. X-ray crystallographic studies of the TBP, TFIIA, and TFIIB proteins bound to the Adenovirus major late promoter have revealed that these factors create a 'protein clamp' that appears to surround almost two full turns of the DNA helix (Werner and Burley, 1996). Next, a preformed RNA Pol II/TFIIF complex binds downstream of TBP through direct interaction by both proteins with TFIIB. TFIIB forms a bridge to the polymerase and sets the approximately 30 bp distance between the TATA box and the transcription start site. TFIIF preferentially recruits a Pol II form with an unphosphorylated carboxy terminal domain (CTD form II-A, see below). TFIIE binds to the complex next through direct interactions with RNA Pol II. Photocrosslinking studies have indicated TFIIE interactions with DNA sequences just upstream of the transcription start site and it has been suggested that it performs a role in promoter melting. Alternatively, it may act as a structural support by bridging the large cleft in RNA Pol II that clasps the DNA helix (Kornberg, 1996). Finally, TFIIH binds through direct interactions with TFIIE and completes the assembly of the preinitiation complex. TFIIH is known to possesses helicase activity for unwinding DNA and kinase activity for phosphorylation of the RNA Pol II CTD. The functions of each component of the preinitiation complex are summarized in Table 1-3.

Factor	Number of Subunits	Function
TFIID	13	Core promoter (TATA) recognition and TFIIB recruitment.
TFIIA	3	Stabilization of TBP binding, stabilization of TAF-DNA interactions, anti-repressor functions.
TFIIB	1	RNA Pol II-TFIIF recruitment, transcription start site selection.
TFIIF	2	Promoter targeting of Pol II, destabilization of non-specific RNA Pol II-DNA interactions.
RNA Pol II	12	Catalytic functions in RNA synthesis, recruitment of TFIIE
TFIIE	2	TFIIH recruitment, modulation of TFIIH helicase, ATPase and kinase activities, direct enhancement of promoter melting (?).
TFIIH	9	Promoter melting using helicase activity, promoter clearance (?), by CTD kinase activity.

Table 1-3. Summary of Human General Transcriptional Initiation Factors. The number of protein subunits that constitute each GTF are indicated along, with their known functions. (Adapted from Roeder, 1996).

The entire assembled preinitiation complex forms contacts with promoter sequences that span over 60 bp DNA. The function of this multiprotein protein complex is to melt the DNA basepair contacts at the initiation site (an ATP-dependent process), initiate the synthesis of the RNA transcript by forming the first phosphodiester bond, and then clear the promoter. The RNA Pol II/TFIIF complex then leaves the initiation complex and begins the elongation phase of transcription. Elongation is characterized by the 5' to 3' migration of transcription bubble and synthesis of the RNA transcript. This process

continues until a transcription termination signal is reached, the transcript is completed, and Pol II/TFIIF recycles to another preinitiation complex.

i) TFIID

The GTF known as TFIID is a large multiprotein complex of 13 subunits (in humans) and includes TBP and its associated TAFs (reviewed in Burley and Roeder, 1996; Roeder, 1996; Tansey and Herr, 1996). TAFs range in size from 15 to 250 kD and are highly evolutionarily conserved from yeast to humans. Crosslinking and mutational studies have nearly pieced together the architecture of the TFIID complex. TFIID has several diverse functions in transcriptional initiation, ranging from structural to catalytic. For example, the sharp 90 degree bend introduced into the TATA region by TBP may function in bringing upstream transcriptional activators into closer proximity to the basal machinery (Werner and Burley, 1996). As well, the largest subunit, TAF250, possesses an intrinsic histone acetylation activity that may be important for chromatin decondensation at the promoter (Mizzen *et al.*, 1996). This subunit is also a kinase that has been shown to phosphorylate TFIIA, TFIIE, and TFIIF, as well as itself (Dikstein *et al.*, 1996).

Interestingly, sequence analyses also identified three TAFs with considerable amino acid sequence similarity to core histone proteins. The *Drosophila* TAF30 and human TAF20 proteins are putative histone H2A homologs. X-ray crystallographic studies revealed that the *Drosophila* TAF42 and TAF62 (human TAF31 and TAF80 homologs) are almost structurally identical to histones H3 and H4, respectively (Xie *et al.*, 1996). These histone-like proteins in the basal transcription machinery may function in the displacement of histones from the promoter sequences or they might secure the DNA/protein contacts between the transcriptional machinery and the promoter. Confirmation of these hypotheses awaits experimental investigation.

Transcription Factor	Activation Domain	TAF
Sp1 Bicoid	Gln-rich	dTAF110
VP-16 p53 NFκB	Acidic	dTAF42* dTAF62*
NTF-1	Ile-rich	dTAF150 dTAF62*
Hunchback		dTAF62*
Retinoblastoma susceptibility gene product		hTAF250
Estrogen Receptor		hTAF30
CAAT transcription factor, YY1, plus multiple other activators		hTAF55

Table 1-4. Compilation of known transcription factors/TAF interactions. Shown are the transcription factors and their corresponding classes of activation domains in addition to the TAFs with which they are known to interact. dTAFs are *Drosophila* proteins and hTAFs are human proteins. '*' indicates TAFs with histone homologies. (Compiled from Verrijzer and Tjian, 1996; Burley and Roeder, 1996).

Sequence analyses have also revealed several well known transcriptional activation-like motifs in certain TAFs, which are believed to be directly involved in interactions with regulatory proteins. Alternatively, several transcription factor activation domains have been observed to interact with certain TAFs. Numerous activator protein-TAF interactions have been described and they have been found to facilitate the recruitment of both TFIID and the other GTFs to the promoter as well as mediate activated transcription (reviewed in Verrijzer and Tjian, 1996). Interestingly, distinct classes of acidic, glutamine-rich, and isoleucine-rich activation domains target distinct TAFs (see Table 1-4). Transcriptional stimulation by

a particular activation domain was found to be dependent on the presence of its corresponding TAFs in the TFIID complex. The finding that distinct activation domains target different TAFs supports a mechanism whereby multiple signals can be integrated by different enhancer bound transcription factors at the promoter complex level.

ii) TFIIH and The Pol II CTD

TFIIH is another complex and multifunctional GTF that has been the focus of intensive study recently. It is comprised of nine evolutionarily conserved protein subunits and its activities include ATPase, kinase, and helicase functions, as well as a nucleotide excision repair function (reviewed in Svejstrup *et al.*, 1996). Two helicase activities have been associated with TFIIH. One unwinds DNA in the 3' to 5' direction and is absolutely required for transcription in vivo, whereas the other unwinds DNA in the 5' to 3' direction and is dispensable for transcription. The latter activity is likely involved in nucleotide excision repair (NER). NER has been found to be an activity of an alternate form of TFIIH that lacks the CTD kinase complex and is exclusively devoted to the DNA repair process.

TFIIH with its CTD kinase activity is an important regulator of transcriptional initiation and elongation. The carboxy terminal domain of the largest RNA Pol II subunit contains highly conserved repeats of a YSPTSPS motif (26 in yeast and 52 in humans). This domain was found to become hyperphosphorylated during the transition from transcriptional initiation to the elongation phase. Phosphorylated Pol II (form II-0) is responsible for RNA synthesis. CTD kinase activity was found to be stably associated with the preinitiation complex and this activity was later ascribed to TFIIH, which has a high specificity for the Pol II CTD (Feaver *et al.*, 1991). TFIIH kinase activity is stimulated by members of the CTD associated 'mediator' proteins (SRB's and SWI/SNF proteins) which are components of the Pol II holoenzyme (reviewed in Bjöklund and Kim, 1996). While the functional significance of CTD phosphorylation is currently unknown, it

has been proposed that it may facilitate the release of Pol II from holoenzyme proteins, such as mediators or GTFs, and allow promoter clearance of the elongation complex.

2. TRANSCRIPTIONAL ACTIVATORS

Each step in the transcription cycle, from preinitiation complex assembly and initiation to promoter clearance and elongation, is controlled by regulatory proteins. The best characterized of these proteins are those that activate the first few steps of transcription. Transcriptional activation is mediated by transcription factors that bind to DNA enhancers or promoter proximal elements. These elements are gene specific arrays of protein binding sites that act as a scaffold upon which higher order nucleoprotein complexes are assembled. The proposed function of these complexes is to increase the recruitment of TFIID and other GTFs to the promoter through secondary stabilizing interactions and to activate transcription, possibly by inducing conformational changes or covalent modifications to the transcriptional machinery (reviewed in Calkhoven and Ab, 1996; Stargell and Struhl, 1996).

Transcription factors are comprised of modular protein domains which are involved in different aspects of their functions. These include domains for DNA binding, dimerization or multimerization, ligand binding, and transcriptional activation. Transcription factors are regulated by very diverse mechanisms. For instance, de novo synthesis, cytoplasmic sequestration, and nuclear translocation may dictate when a factor may act in the nucleus. Moreover, phosphorylation, glycosylation, or ligand binding may serve to increase the DNA binding activity or the transcriptional activation potential of a given factor.

Transcription factors generally employ one of several different classes of transcriptional activation domains. These include acidic, glutamine-rich, isoleucine-rich, or proline-rich amino acid sequences. Activation domains are believed to function via direct protein-protein interactions with the general transcription machinery, or via the association

with an adaptor protein that bridges these interactions. For instance, several transcription factors have been shown to physically interact with TFIIA, TFIIB, TFIID, TFIIF, and TFIIH but the functional relevance of these interactions is largely unknown. One possibility that has been proposed is that contact with activation domains might enhance the intrinsic enzymatic activities of the basal transcription apparatus, such as the kinase activities of TFIIH or TAF250 (Goodrich *et al.*, 1996).

Transcription factors play a vital role in cell-specific gene induction but how they achieve such specificity is poorly understood. Many transcription factors are encoded by multigene families or they are composed of functionally diverse protein products created by alternate splicing of a single transcript or from alternate translation start sites in a single mRNA. Commonly, these protein products form heteromeric complexes that are composed of different combinations of related subunits or contain subunits from other transcription factor families. For example, the AP1 complex is composed of either Jun/Jun homodimers or Jun/Fos heterodimers. Both Jun and Fos are encoded by a family of related genes. These proteins also form heteromeric complexes with NFATc and members of the ATF/CREB family of transcriptional activators. Each of these may have differential DNA binding specificities or transcriptional activation potentials, which may be subtle or quite dramatic. Many transcription factor subunits are ubiquitously expressed, but limiting the expression of certain factors to one or a few tissues promotes tissue specificity in gene regulation. As well, DNA binding sites govern specificity by displaying slight variations of transcription factor binding sequences that function in the recruitment of only a very specific subset of transcription factors that bind preferentially to that site.

3. CHROMATIN AND DNASE1 HYPERSENSITIVE SITES

Within the nucleus, eukaryotic DNA is bundled into highly compact and ordered nucleoprotein structures, which in general terms is referred to as chromatin. Chromatin is composed of duplex chromosomal DNA, plus all of its associated proteins in the nucleus. Chromatin exists in two general forms, euchromatin and heterochromatin. Euchromatin refers to areas where the DNA is relatively loosely associated with chromatin proteins and generally contains the expressed genes within a cell. Heterochromatic areas contain DNA that is highly condensed and tightly associated with chromatin proteins and these regions contain non-transcribed DNA.

a. Histones

The major protein component of chromatin is the histones. DNA is wrapped twice around a globular core of eight histones proteins (H2A, H2B, H3, and H4)$_2$ in a left hand superhelix to form a nucleosome, the unit repeat of chromatin (reviewed in Paranjape *et al.*, 1994; Wolffe and Pruss, 1996). Each nucleosome contains the histone octamer, approximately 180 bp of DNA, and one copy of histone H1 which binds to the internucleosomal DNA. Nucleosomes associate into a coiled higher order structure called the 30 nm chromatin fiber. The result of histone association is a 40 to 50 fold compaction of DNA and the concealment of vast amounts of nucleotide sequence within the structure.

Transcription in vivo is inhibited by the general repressive effects of chromatin. The basal transcription machinery and its activators must overcome this repression by displacing chromatin proteins in order to gain access to the DNA. One method of antirepression involves covalent modification of the histone N-terminal tails (reviewed in Paranjape *et al.*, 1994; Wolffe and Pruss, 1996). The N-terminal tails of histones H3 and H4 are positively charged and protrude out from the globular core of the histone octamer where they may interact with the DNA or other proteins. Acetylation of lysine residues within these tails is proposed to lower their positive charge and disrupt interactions with

negatively charged DNA. The presence of hyperacetylated histone tails has been observed to correlate with actively transcribed genes. This could be due in part to the acetyltransferase activity of TAF250 but it could also reflect the action of other general histone acetylases such as Gcn5 (Brownell *et al.*, 1996). For example, histone hyperacetylation was detected across the entire 33 kb DNase1 sensitive domain of the chicken β-globin locus but not in regions beyond (Hebbes *et al.*, 1994). This indicates that acetylation may alter chromatin structure by making it less condensed and more accessible to the transcription proteins. Phosphorylation may also play a role in chromatin structure. One of the observed responses to stimulation of cells with growth factors and phorbol esters was the rapid phosphorylation of histone H3 tails on specific serine residues (Mahadeven *et al.*, 1991). However, how these histone modifications are targeted to specific chromatin domains remains unknown.

b. SWI/SNF

Eukaryotes have evolved elaborate mechanisms in order to overcome the repressive effects of histones and other chromatin associated proteins. One of these mechanisms involves a very large two Megadalton complex of proteins referred to as SWI/SNF. This complex was first identified in yeast as an activator of many inducible genes and evolutionarily conserved components have since been identified in *Drosophila*, mice, and humans. It was first observed to facilitate the binding of the yeast GAL4 transcription factor to reconstituted nucleosomal DNA in vitro by altering nucleosome/DNA interactions in an ATP-dependent fashion (Imbalzano *et al.*, 1994; Kwon *et al.*, 1994). How the SWI/SNF complex disrupts nucleosomes is unclear but it is believed to interact with components of the histone octamer, as certain histone mutations can suppress mutations in SWI/SNF (Hirschhorn *et al.*, 1992; Kruger *et al.*, 1995). In addition to interactions with histones, studies have indicated that the SWI/SNF complex introduces positive supercoils into plasmid DNA and has a high affinity for structured DNA, such as four way junctions

(Quinn *et al.*, 1996). Interestingly, the purified SWI/SNF complex forms distinct in vitro footprints on DNA fragments but these interactions appear to be non-sequence-specific.

Components of the SWI/SNF complex have been purified from several organisms. An important connection between the SWI/SNF complex and activated transcription was discovered when Western blotting and immunoprecipitation studies showed that SWI/SNF proteins directly associate with the SRB proteins that are an integral component of the RNA Pol II holoenzyme (Wilson *et al.*, 1996). SRB proteins, also known as the 'mediator' complex, associate with the CTD of RNA Pol II and enhance TFIIH-dependent phosphorylation of the CTD, as well as stimulate both basal and activated transcription (reviewed in Björklund and Kim, 1996). The discovery of nucleosome remodeling activities associated with the RNA Pol II holoenzyme proposes an intrinsic anti-repression mechanism for the transcriptional machinery to counter the inhibitory effects of chromatin. Interestingly, purification of the SWI/SNF complex from several different mammalian cell lines revealed that it is somewhat heterogeneous with respect to subunit composition in different cell types (Wang *et al.*, 1996). This implies that different cell types or tissues may harbor specialized SWI/SNF complexes that could be important for chromatin remodeling in tissue-specific genes.

c. DNase1 Hypersensitive Sites

Many DNA binding proteins have been observed to physically distort DNA (reviewed in Werner and Burley, 1997). This bending, up to 130 degrees in the case of the T cell-specific transcription factor LEF-1, is believed to bring upstream and downstream regulatory factors into closer proximity and to facilitate the displacement of DNA from nucleosomes. However, whether the binding of transcription factors is dependent on, or results in, nucleosome displacement is still the subject of debate. Nucleosome displacement generates a short stretch of DNA, approximately 200 bp in length, that is exposed and susceptible to nicking by nucleases in intact nuclei (reviewed in Gross and

Garrard, 1988). DNase1 is a commonly employed nuclease used to probe for these sequences that generally correlate with the location of promoters and enhancers in the vicinity of actively transcribed genes. Some hypersensitive sites are constitutive and likely reflect structured DNA, but many are inducible and appear in response to particular stimuli or cellular activation. More precisely, they may appear in a tissue-specific or developmental stage-specific manner.

DNase1 hypersensitive (DH) sites that are important for developmental or tissue-specific gene expression often contain binding sites for both tissue-specific and ubiquitously expressed transcription factors. Several closely linked DH sites exhibiting tissue and developmental stage-specific appearance have been characterized in the β-globin locus. Four erythroid-specific DH sites located 5' of the globin gene cluster have been found to direct the tissue and developmental stage-specific expression of each globin gene (embryonic, fetal, and adult) (Engle, 1993; Felsenfeld, 1993; Frazer *et al.*, 1993; Lowrey *et al.*, 1992; Strouboulis *et al.*, 1992). Detailed examination of the individual sites (HS1, HS2, HS3, and HS4) revealed multiple transcription factor binding sites for both erythroid-specific and ubiquitously expressed transcription factor proteins (Bresnick and Felsenfeld, 1993; Gong and Dean, 1993; Hardison *et al.*, 1993; Hug *et al.*, 1992; Ney *et al.*, 1993). For example, DNase1 footprint analysis of HS3 and HS4, active in the embryonic and adult stages respectively, revealed binding sites for the ubiquitous transcription factors AP1 and Sp-1, and for the erythroid-specific factors GATA-1 and NF-E2.

Studies investigating the relationship between transcription factor binding and the formation of DH sites have revealed that site formation is an all or nothing phenomenon and that the probability of hypersensitive site formation in a particular cell decreases if one or more transcription factors are limiting (Felsenfeld *et al.*, 1996; Rothenberg and Ward, 1996). For example, the wild type chicken β-globin enhancer is hypersensitive in 91% of a population of avian erythrocytes but the mutation of two GATA-1 binding sites was found

to reduce the number of erythrocytes forming hypersensitive sites to 39% (Boyes and Felsenfeld, 1996). In a similar study involving the IL-2 gene enhancer, the cyclosporin induced inhibition of DNA binding by calcium-dependent transcription factors resulted in the complete absence of DNA binding by any other transcription factors to the endogenous locus (Garrity *et al.*, 1994). These examples of enhancer occupancy suggest that transcription factors additively increase the probability of DH site formation and nucleosome displacement, whereas binding by individual factors is less favored. Consequently, an enhancer with binding sites for both tissue-specific and ubiquitous transcription factors would undoubtedly be active in the appropriate cell type, in response to the appropriate stimuli, when all of the necessary transcription factors are present and in their activated forms.

III. REGULATION OF GENE EXPRESSION IN T LYMPHOCYTES

1. LYMPHOID-SPECIFIC TRANSCRIPTION FACTORS

Many lineage-specific genes are transcriptionally induced by similar nuclear factors. Studies of the enhancer regions of T cell-specific genes, such as the cytokines and the T cell receptors molecules, have led to the identification of several lymphoid-specific transcription factors. These factors are expressed at discrete stages of hematopoietic development where they direct the terminal differentiation of T cells by coordinating the stage-specific expression of lymphoid-restricted genes (reviewed in Clevers and Grosschedl, 1996; Fitsimmons and Hagman, 1996; Shivdasani and Orkin, 1996). Moreover, they direct T cell-specific gene expression by combining with ubiquitous transcription factors to enhance activation. A summary of these transcription factors is presented in Table 1-5. Recent experiments in various transcription factor knockout mice have facilitated the identification of key transcriptional regulators of pivotal steps in

Transcription Factor	Alternate Names	DNA binding domain	DNA binding sequence	Potential Gene Targets	Hematopoietic Expression
c-Myb		Myb	T/CAACG/TG	CD4, TCR-δ, γ	Pre-B and T cells
Core Binding Factor α (CBFα)	AML1 PEBP2α	Runt	TGYGGT	GM-CSF, IL3, CSF-R, TCR α, β, γ, δ, Granzyme B	B and T cells
Core Binding Factor β (CBFβ)	PEBP2β	None	Associates with CBFα	Same as above	Ubiquitous
Ets1	c-Ets-1	Ets	A/GCC/AGGA/TGT	TCR α, β, nur77, CD4, Igμ, Igκ3', bcl-2, bcl-x	B and T cells
GATA2		Zinc Finger	A/TGATAA/G	CD3δ, TCR α, β, γ, δ	Hematopoietic cells
GATA3		Zinc Finger	A/TGATAA/G	CD8α, CD2, TCR α, β, δ	T cells
Ikaros		Zinc Finger	GGGAA/T	CD3δ, granzyme B	T and B cells
LEF1	TCF-1α	HMG-1/2	TTCAAAG	TCR α, β, δ; CD4	T cells and pre-B cells
Sox-4		HMG-1/2	A/TA/TCAAAG/T	CD3ε, CD2	T cells and immature B cells
TCF1		HMG-1/2	A/TA/TCAAAG	TCR α, β, δ; CD3ε, CD4, CD8α, IL-4, IL-13	T cells
NF-κB/c-Rel	Rel	Rel	GGGA/GNNC/TC/TCC	IL-2, IL-6, GM-CSF, ICAM-1	Ubiquitous

Table 1-5. Biochemical properties of several families of transcription factors expressed in T cells. Shown are a number of transcription factors that have been implicated in hematopoietic development and lymphoid-specific gene expression. The alternate names of the transcription factors, their DNA binding domain classifications, consensus DNA binding sequence, examples of lymphoid-specific target genes, as well as patterns of expression are shown. (Adapted from Clevers and Grosschedl, 1996; Fitsimmons and Hagman, 1996; Shivdasani and Orkin, 1996).

differentiation. For instance, it was found that disruption of the transcription factors GATA-2, GATA-3, or Myb affected the development of multiple hematopoietic lineages whereas the disruption of the transcription factor Ikaros resulted in the failure to develop only the lymphoid lineage. The biochemical details of two important lymphoid-specific transcription factors, the core binding factor and Ikaros, will be discussed.

a. Core Binding Factor

The core binding factor (CBF), also known as the polyoma enhancer binding protein (PEBP2) and the acute myeloid leukemia protein (AML1), was first identified as a factor that bound to the enhancer cores of several lymphoma associated mammalian type C retroviruses (Golemis *et al.*, 1990) and has since been recognized as a regulator of many T cell-specific genes. These include the IL-3 gene (Taylor *et al.*, 1996), the CD36 gene (Armesilla *et al.*, 1996), the macrophage colony stimulating factor (M-CSF) receptor promoter (Zhang *et al.*, 1996), and the T cell receptor (TCR) genes α, β, γ and δ (Hernandez-Munain *et al.*, 1995; Hsiang *et al.*, 1993; Hsiang *et al.* 1995; Prosser *et al.*, 1992; Sun *et al.*, 1995; Wang and Speck, 1992; Wotton *et al.*, 1994). CBF expression is first detectable at very early stages of T cell development and is maintained in mature T cells. This expression pattern implicates CBF as a key transcriptional regulator of T cell-specific gene expression during both T cell development and activation.

Core binding factor (CBF) is composed of two structurally unrelated subunits; the CBFα subunit, which binds to DNA, and the CBFβ subunit, which associates with CBFα and augments its DNA binding activity. The CBFα subunit is encoded by three related but distinct genes; CBFαA (AML3/PEBPα2A), CBFαB (AML1/PEBPα2B), and CBFαC (AML2/PEBPαC), each of which generates multiple protein isoforms from alternatively spliced mRNAs (Bae *et al.*, 1994; Levanon *et al.*, 1994; Miyoshi *et al.*, 1995). The different isoforms of CBFα subunits were found to possess differential DNA binding and transcriptional transactivation activities (Bae *et al.*, 1994). These proteins all contain an

evolutionarily conserved DNA binding and heterodimerization domain termed 'runt' as it is similar to the *Drosophila* Runt proteins (Kagoshima *et al.*, 1993). The αA gene appears to be expressed only in T cells (Ogawa *et al.*, 1993) whereas the αB gene is expressed in both B cells and thymocytes (Bae *et al.*, 1993). Conversely, the αC gene is ubiquitously expressed (Bae *et al.*, 1995). The αB protein was recently determined, by transgenic knockout experiments, to be an essential factor in liver derived hematopoietic development. This indicates that the different isotypes of CBF do not function in a redundant manner (Okuda *et al.* 1996). The murine CBFβ subunit is comprised of three distinct but related proteins, each of which is translated from differentially spliced transcripts encoded by a single gene (Wang *et al.*, 1993).

CBF activity appears to be activated by at least two mechanisms; nuclear translocation and phosphorylation. First, the CBFα subunit exhibits relatively weak DNA binding in the absence of the β subunit, but the αβ heterodimer efficiently binds to DNA. Immunohistochemical staining of ectopically expressed CBFα and CBFβ proteins in NIH 3T3 cells revealed that CBFα is a nuclear protein, whereas CBFβ is localized mainly in the cytoplasm (Lu *et al.*, 1995). Interestingly, the β subunit localized to the nucleus in cells which expressed either an N- or C-terminally truncated α subunit. This indicated that β is passively translocated into the nucleus and that association with the α protein may be prevented by distinct domains of the α subunit. These α domains may require some form of modification before associating with β. Recently, one of the CBFα proteins (CBFαB) was identified as a substrate for ERK kinases (Tanaka *et al.*, 1996). Activated ERK was shown to phosphorylate two specific serine residues in the C-terminal domain of CBFαB both in vitro and in vivo. Although the in vivo expressed proteins exhibited a substantial level of transcriptional transactivation in the absence of ERK, phosphorylation resulted in a three to four fold increase in the expression of a reporter gene linked to the TCRβ gene enhancer. An attractive model is that the ERK-dependent phosphorylation of CBFα relieves its heterodimerization inhibition and results in the nuclear translocation and

association of the β subunit. Confirmation of this hypothesis, or the identification of other modes of CBF activation, awaits future investigations.

The most intriguing feature of CBF is that it often functions in combination with other transcription factors. Cooperative DNA binding and functional interactions between CBF and a variety of other transcription factors have been observed in several different T cell-specific enhancers. For example, CBF and the transcription factor Ets-1 displayed enhanced cooperative DNA binding to the TCRα and TCRβ enhancers in vitro (Giese *et al.*, 1995; Wotton *et al.*, 1994). In the TCRβ enhancer, this interaction appeared to be unperturbed by changes (5 or 10 bp) in the relative spacing or orientation of either transcription factor binding site. The proteins appeared to function as a unit in the enhancer as both intact binding sites were required for efficient transcriptional activation (Sun *et al.*, 1995). In the TCRδ enhancer, CBF and the transcription factor c-Myb were observed to function as an inseparable transcriptional activating unit (Hernandez-Munain and Krangel, 1995). Although cooperative DNA binding by the two factors was not examined in this study, an intact c-Myb binding site and the close juxtaposition (4 bp apart) of the CBF and c-Myb binding sites were absolutely required for the transcriptional activation of a reporter gene in transient transfection assays. Similarly, transcriptional activation of the macrophage colony stimulating factor (M-CSF) receptor promoter was mediated by a CBF/CCAAT enhancer binding protein (C/EBP) complex (Zhang *et al.*, 1996). Mutations in either DNA binding site, or the separation of the closely juxtaposed binding sites (3 bp) by 5 or 10 bp, completely abrogated the transcription of a reporter gene in transfection assays. The mechanism of transactivation by CBF and other transcription factors is not known. It is possible that CBF facilitates the recruitment of other transcription factors to enhancers. Alternatively, these complexes could form a composite surface that interacts with other proteins, such as other transcription factors or the basal transcriptional machinery.

b. Ikaros

Ikaros is a zinc finger DNA binding protein that has been proposed to function as a master regulator of lymphoid development. It is expressed exclusively in lymphocytes and hematopoietic progenitor cells and Ikaros knockout mice exhibit a complete absence of T and B lymphocytes and NK cells (Georgopoulos *et al.*, 1994). Ikaros was first identified as a transcription factor that bound to a G-rich binding sequence the CD3δ enhancer and has since been implicated in regulation of other T cell-specific genes including CD4, CD2, IL-2, IL-2R, TCRα, TCRβ, CD3γ, and CD3ε (Molnar and Georgopoulos, 1994). A single Ikaros gene encodes a family of five proteins from differentially spliced mRNAs (Ik-1 through Ik-5) with overlapping, but distinct, DNA binding specificities and differential transcriptional transactivation properties (Molnar and Georgopoulos, 1994; Molnar *et al.*, 1996). Each isoform of Ikaros contains a unique combination of four distinct zinc finger modules, that define their DNA binding specificities. Additional zinc finger motifs, in the distal C-terminus of all isoforms, have recently been demonstrated to mediate interactions between Ikaros proteins. Mutations in these domains ablate Ikaros protein-protein interactions and abrogate the ability of these proteins to bind DNA and activate transcription (Sun *et al.*, 1996).

The differential expression patterns and functions of the Ikaros isoforms may substantiate their role in lymphoid development and activation (Molnar and Georgopoulos, 1994). Ik-1 and Ik-2 are the predominant forms at all lymphoid developmental levels and are the strongest transcriptional activators. Ectopic expression and immunohistochemical staining in NIH 3T3 cells revealed that these proteins localize to the nucleus. Ik-3 and Ik-5 are also expressed throughout lymphoid development but are present at relatively low levels, while Ik-4 is only expressed in early thymocytes. Transiently expressed Ik-3 and Ik-4 were weak transcriptional activators of T cell-specific enhancers in NIH 3T3 cells. However, this observation may have been due to the fact that fluorescence in situ hybridization revealed that these proteins were localized to the cytoplasm in this cell type.

Whether these Ikaros isoforms reside in the cytoplasm, or are weak transcriptional activators in lymphoid cells, remains to be determined. Overall, T cell-specific gene expression may be differentially regulated by different subsets of Ikaros proteins at every developmental level.

2. ACTIVATION-DEPENDENT T CELL-SPECIFIC GENES

a. The IL-2 Gene

By far, the best characterized of the T cell-specific genes is the IL-2 gene, which is rapidly induced in Th cells following TCR stimulation and CD28 coreceptor ligation, or in response to treatment with calcium ionophores and phorbol esters (Smith, 1980; Gillis and Watson, 1980). The IL-2 promoter is a target for several ubiquitous and lymphoid-specific transcription factors, which include NF-AT, NF-AP1, NFκB, AP1, pOCT, CD28 response element binding protein, and possibly Ikaros. IL-2 enhancer binding by each of these factors has been documented by in vivo footprinting analysis (Chen and Rothenberg, 1993). As well, the stimulation-responsive transcriptional transactivation activities of most factors have been investigated in transient transfection experiments (Durrand *et al.*, 1988). Each binding site has been disrupted by in vitro mutagenesis and their relative contributions to overall promoter activity has been studied in T cell clones and in activated primary human lymphocytes by transfection analysis (Hughes and Pober, 1996). Most of the transcription factors were found to be important for reporter gene expression in T cells, whereas a few, such as the CD28RE, the distal NF-AT, and the distal AP1 elements, had little effect. Mutations in the NFκB, pOCT, proximal AP1, or the proximal NF-AT binding sites reduced promoter activity to less than half of wild type levels. Interestingly, the Jurkat T cell line was much more sensitive to mutations in the NF-AP1 and distal NF-AT binding sites compared to primary lymphocytes. This suggests that primary lymphocytes and T cell clones contain different complements of activated transcription factors. The study of the endogenous IL-2 promoter has revealed that the presence of all

activated transcription factors is required to assemble an active enhancer complex in the context of chromatin. For example, the treatment of EL4 cells with cyclosporin inactivated the calcium-dependent transcription factors, but it led to the complete absence of binding by any of the other transcription factors to the endogenous promoter. Thus, enhancer complex assembly appeared to be dependent on the activation of all the necessary transcription factors, which in turn must be activated by all of the obligatory signal transduction pathways, before the gene is activated (Garrity *et al.*, 1994).

b. The Perforin Gene

In comparison to IL-2, the regulation of CTL-specific gene expression has not been as well documented. The primary reason has been the lack of suitable CTL clones in which to study the induction of CTL-specific genes, such as perforin or the various granzymes. Most CTL clones constitutively express these genes, making the study of transcriptional induction impossible. As well, CTLs have typically been resistant to standard transfection methods, presumably due to inherent membrane protective mechanisms that prevent CTL from being killed by their own lytic molecules. Nonetheless, the development of transfection protocols for several CTL lines has made it possible to identify the regulatory sequences that are important for the transcription of perforin and granzymes and to begin to elucidate the mechanisms that underlie their cell-specific expression.

The murine perforin gene is comprised of three exons that span approximately 7 kb of chromosome 10 (reviewed in Podack *et al.*, 1991; Liu *et al.*, 1995). The gene is expressed in all functional killer cells, such as $CD4^+$ and $CD8^+$ CTL and NK cells, and both transcript and protein levels correlate well with killing potential. However, the mechanisms of transcriptional regulation of the perforin gene have been difficult to interpret. Lichtenheld and Podack (1992) reported that the 5 kb of 5' flanking region is composed of multiple positive and negative regulatory elements that are differentially active in different cell types. The minimal promoter (-120 to +2) was active in all cell types examined by

transient transfection reporter gene analysis. However, high levels of cell type-specific reporter gene expression were observed with the entire 5 kb of flanking region. Deletion analysis of sequences between -5 kb and -120 bp revealed a bewildering pattern of positive and negative activities, that differed between perforin expressing and non-expressing cell types. Overall, perforin expression and CTL-specificity appeared to be governed by the entire 5 kb of flanking region and the sequences therein. This flanking region was later examined for the ability to confer cell type-specific expression of a reporter gene in transgenic mice (Lichtenheld *et al.*, 1995). It was found that killer cell-specific expression did occur, but the reporter gene was expressed in both immature and resting T cells, whereas the endogenous gene was not. Therefore, additional regulatory sequences exist elsewhere in the gene that are involved in repressing perforin expression until the mature T cell is activated.

In a recent study by Youn *et al.* (1996), seven CTL-specific DNase1 hypersensitive sites were mapped within the murine genomic perforin gene. One DH site existed 7 kb upstream of the promoter, five were dispersed within 2 kb 5' of the transcription start site, and one was present in intron 2. The composition of the most proximal of these DH sites (at approximately -60 bp) was examined. Four potentially important transcription factor binding sites were identified that corresponded to Sp1, Sp1-related (possibly Ikaros), Ets, and GATA-like sequences. Mutational analysis of the first three binding sites revealed that the abolishment of the individual sites resulted in a 2.5 to 3.5 fold reduction in the transcriptional activity of both the proximal perforin promoter (-73 bp) and a longer, highly expressed perforin promoter (-795 bp) in CTLs. However, neither of these wild type promoters were found to be appreciably CTL-specific. Clearly, more studies are required to elucidate the importance of the other DH sites, and whether they are involved in the CTL-specificity of perforin transcription.

c. The Granzyme B Gene

Granzymes are a highly conserved family of CTL and NK cell-restricted serine proteinase genes. Six of the seven murine granzyme genes (B through G) are clustered on chromosome 14 (Crosby *et al.*, 1990), while the granzyme A gene is located on chromosome 13 (Mattei *et al.*, 1987). The organization of the granzyme locus has recently been determined (Pham *et al.*, 1996). Granzyme B is located at the 5' end of the cluster, followed by granzymes C, F, G, D, and E (in the 3' direction). The orientation of the genes is not yet known, although, all three of the human granzyme genes were found to be transcribed in the same direction. Each gene in the locus consists of five highly conserved exons, which suggests that the granzyme locus arose from gene duplication events during evolution (Prendergast *et al.*, 1991; Prendergast *et al.*, 1992a). However, the 5' flanking sequences appear to be quite divergent, and it became evident that the granzymes were regulated by different transcriptional mechanisms when it was found that each gene displayed different kinetics of induction in response to various stimulation conditions (Prendergast *et al.*, 1992b).

Of all the granzyme genes, granzyme B is unequivocally the best characterized in terms of transcriptional regulation. Like perforin, granzyme B is only expressed in $CD4^+$ and $CD8^+$ CTL and NK cells, however, there does not appear to be any obvious sequence similarities between the granzyme B and perforin promoters. Both granzyme B transcript and protein levels have been correlated with cytolytic activity (Prendergast *et al.*, 1992b). Granzyme B transcription peaks after two days of stimulation with concanavalin A or αCD3 and steady state transcript levels peak on day two, three, or four, depending on the type of stimulus issued (Lobe *et al.*, 1989; Prendergast *et al.*, 1992b).

Most of the granzyme B promoter studies performed to date have focused on the expression of the human gene. The human granzyme B 5' flanking sequences (-1170 bp to +31) have been analyzed for the ability to confer CTL-specific gene expression on a reporter gene in transgenic mice (Hanson *et al.*, 1991). These sequences directed the T

cell-specific and activation-dependent expression of the transgene with kinetics that were very similar to the endogenous murine granzyme B gene. This study indicated that the tissue specificity and induction of granzyme B are controlled by sequences that are contained within the 5' flanking region.

Transient transfection analyses, with deletion fragments of the human granzyme B 5' promoter sequences, have localized the majority of the transcriptional activity to within 150 bp of the transcription start site (Haddad *et al.*, 1993; Hanson *et al.*, 1993). Within this proximal promoter region there exists consensus binding motifs for the transcription factors AP1 and CRE. Both binding sites were shown to be required for the transcriptional activation of the granzyme B promoter in transient transfection assays in TPA and dibutyryl cAMP stimulated PEER cells (Hanson *et al.*, 1993). These cells possess γδTCRs and only express granzyme B upon stimulation. An inducible DNase1 hypersensitive site was identified in the endogenous granzyme B promoter in stimulated PEER cells that extends from approximately 30 to 400 nucleotides upstream of the transcription start site and corresponds to the proximal promoter sequences (Hanson *et al.*, 1990). As well, putative binding sites for lymphoid-specific transcription factors Ikaros and core binding factor have been identified within this region (Warginer *et al.*, 1995).

The granzyme B proximal promoter sequences are highly conserved between mice and humans. As well, the relative spacing of the AP1, CBF, and CRE transcription factor binding sites are identical between the two promoters. Transient transfection analysis of murine granzyme B promoter fragments revealed that a 243 bp proximal promoter was highly active in a CTL cell line, but not in L cell fibroblasts or EL4 thymoma cells (Frégeau and Bleackley, 1991). Alternatively, a smaller 112 bp fragment was inactive in CTL. This suggested that the region between -112 and -243 was responsible for the high levels of expression and CTL-specificity observed from the 243 bp promoter.

In both the human and murine genes, conserved proximal promoter sequences have been identified which are important for the T cell-specificity and the activation-dependent

expression of the granzyme B gene. Identifying the transcription factors that target the murine granzyme B gene will provide key insights into the mechanisms of CTL-specific granzyme B activation and provide valuable biochemical clues about the nature of the signaling events that must occur to achieve this end.

THESIS OBJECTIVES

The granzyme B gene is induced in cytotoxic T lymphocytes in response to antigenic stimulation. Several distinct signal transduction pathways are activated that are integrated in the nucleus in the form of a particular subset of activated transcription factors that converge on the granzyme B promoter and activate its transcription. By studying these transcription factors we can begin to understand the molecular events that are involved in the transmission of signals from the TCR to the nucleus upon activation of T cells.

Investigations into the transcriptional control of the murine granzyme B gene began in our laboratory ten years ago. A major positive control element was identified within the 243 nucleotides that flank the promoter. However, the exact locations of important *cis*-acting regulatory sequences, or the identity of the transcription factors that bind to these elements, were only speculative at the beginning of this work. The studies described in this thesis localize the important 5' regulatory elements involved in granzyme B transcription and identify the transcription factors that bind to each of these sequences in activated cytotoxic T cells. To assess the importance of each transcription factor in the regulation of the granzyme B gene, each protein binding site was disrupted by site directed mutagenesis to investigate their role in promoter function. Importantly, primary activated $CD8^+$ murine lymphocytes were employed as the principal experimental system in which to examine the activities of transiently transfected promoter fragments. As well, the status of the endogenous granzyme B promoter was probed before and after primary lymphocyte activation. Finally, granzyme B expression was investigated in $CD4^+$ primary activated T cells.

CHAPTER 2

MATERIALS AND METHODS

I. MATERIALS

1. Antisera and Purified Antibodies. The hamster anti-mouse αCD3ε monoclonal antibody is produced by 145-2C11 cells obtained from Dr. J. Bluestone. It was partially purified from culture supernatants by ammonium sulphate precipitation as described (Leo *et al.*, 1987). The αCD28 antibody was purchased from PharMingen. The rat anti-mouse CD4, rat anti-mouse CD8, rat anti-mouse CD4 phycoerythrin-conjugated, and the rat-anti-mouse CD8 fluorescein isothiocyanate-conjugated monoclonal antibodies were purchased from Serotec. The affinity purified rabbit anti-mouse granzyme B antibody was kindly provided by Dr. Dorothy Hudig. Antisera to the PEBP2 α and β subunits was kindly provided by Yoshiaki Ito. Anti-αA1N35 serum reacts with the α subunit, and rabbit anti-β2 serum reacts with the β subunit of the PEBP2 complex. Purified c-fos (K-25) and c-jun/AP-1 (D) antibodies were obtained from Santa Cruz Biotechnology.

2. Animals. BALB/c mice were obtained from the University of Alberta Laboratory Animal Services department

3. Enzymes. Most restriction endonucleases, DNA ligase, Klenow fragment of *E. Coli* DNA polymerase 1, Taq polymerase, proteinase K, and T4 polynucleotide kinase were purchased mainly from Gibco/BRL Life Technologies and Boehringer Mannheim. Avr II was purchased from New England Biolabs. DNase1 was obtained from the Sigma chemical company.

4. Radioactive Isotopes. The radioactive nucleotides α[^{32}P]-dCTP [deoxycytidine 5'-triphosphate tetra-(triethylammonium) salt and γ[^{32}P]-ATP

[deoxyadenosine 5'-triphosphate tetra-(triethylammonium) salt] were purchased from the New England Nuclear company.

II. METHODS

1. Cells and Cell Culture. Primary splenocytes were obtained from six to twelve week old Balb/c mice. Spleen tissue was ground through a fine wire screen in RHFM/IL-2 medium and the cells were pelleted. Red blood cells were lysed with buffered ammonium chloride lysis buffer. The IL-2 dependent cytotoxic T cell line MTL 2.8.2 was generated from CBA/J mice as described (Bleackley *et al.*, 1982). The antigen and IL-2 dependent CTL21.9 (called Type1) line was generated as described (Havele *et al.*, 1986). EL4 is an IL-2 producing T lymphoma cell line (Paetkau *et al.*, 1986) and L cells are a mouse fibroblast line. All cells were cultured in RHFM [RPMI-1640 supplemented with 20 mM HEPES (pH 7.5), 100 μM β-mercaptoethanol, and 10% fetal bovine serum (5% for L cells)] at 37° C in 5% CO_2. Type1 cells, MTL 2.8.2 cells, and primary splenocytes were cultured in the presence of 60 U/ml human recombinant IL-2. Primary splenocytes were stimulated with 5-10 μg/ml concanavalin A (Sigma), 1:300 to 1:1000 (determined empirically) dilution of αCD3, and 1:2000 dilution of αCD28, alone or in combination. EL4 cells were stimulated with 10 ng/ml phorbol myristate acetate (PMA) (Sigma) and 4 μM ionomycin (Sigma).

2. Cell Separation. $CD8^+$ and $CD4^+$ primary splenocytes were immunomagnetically isolated from whole splenocyte populations by incubation with rat anti-mouse αCD8 or αCD4 primary antibodies (obtained from Serotec) followed by incubation with sheep anti-rat secondary antibodies conjugated to magnetic Dynabeads® (Dynal®). Bead labeled conjugates were separated from the whole splenocyte culture by repeated magnetic separation and washing steps. Enriched T cell and $CD4^+$ and $CD8^+$ T cell subsets were obtained by passage over Cellect™ immunocolumns (Biotex Laboratories Inc.) designed to retain B cells, adherent cells, and the undesired T cell subset (if required).

The purity of the selected cell populations were routinely confirmed by flow cytometry analysis.

3. Transfections and Luciferase Assays. Transient transfections were performed using a DEAE dextran transfection procedure optimized for cytotoxic T cells (Fregeau *et al.*, 1991) with variations for the different cell types. Primary splenocytes were cultured at $3.0x10^6$ cells/ml in RHFM plus 60 U/ml IL-2, 1:500 dilution αCD3, and 5 μg/ml concanavalin A for 20 hours prior to transfection. Basically, $1.0x10^7$ logarithmically growing cells (T cell lines) or $2.0x10^7$ (whole splenocytes) were washed twice in serum-free medium and resuspended in 1.0 ml TBS (25 mM Tris-HCl [pH 7.5], 137 mM NaCl, 5 mM KCl, 0.6 mM Na_2HPO_4, 0.7 mM $CaCl_2$, and 0.5 mM $MgCl_2$ [pH to 7.0]) with 500 μg/ml DEAE dextran (Sigma), 15 μg covalently closed circular luciferase reporter plasmid, and 5 μg β-galactosidase control plasmid. The DNA was adsorbed for 15 minutes at room temperature. Cells were washed twice in serum-free medium and cultured at $5x10^5$ cells/ml (cell lines) or $1.0x10^6$ cells/ml (splenocytes) in RHFM + 60 U/ml IL-2 and incubated at 37° C in 5% CO_2. Primary splenocytes were stimulated with additional αCD3 and concanavalin A following transfection. The cells were harvested after 48 hours, washed twice in phosphate buffered saline (PBS), lysed in Triton lysis buffer (1% Triton X-100, 25 mM glycylglycine, pH 7.8, 15 mM $MgSO_4$, 4 mM EGTA, 1 mM DTT). L cells were transfected as described (Seldon, 1992).

4. Plasmids. Granzyme B promoter fragments (from our C11 gene clone) (Frégeau and Bleackley, 1991) and serpin 2A promoter fragments were obtained by restriction enzyme digestion or PCR amplification. All fragments were inserted upstream of the promoterless luciferase reporter gene in either the p19LUC (De Wet *et al.*, 1987) or the pGL2 (Promega) plasmids and orientation was confirmed by sequencing or directional primer amplification. The two viral promoter driven plasmids RSVLUC (De Wet *et al.*, 1987) and pSVGL2 (Promega) were used as positive controls. RSVLUC contains the luciferase gene under the control of the Rous Sarcoma viral promoter and pSVGL2

contains the luciferase gene under the control of the SV40 viral promoter. Two β-galactosidase control plasmids were used as transfection standards. SVβ-gal has the bacterial β-galactosidase gene under the control of the SV2 viral promoter (Promega) and 906 (from A. Puschel) has the bacterial β-galactosidase gene under the control of a human actin promoter. Granzyme B reporter gene plasmids were grown in the presence of ampicillin selection in DH5α *Escherichia coli* and high quality supercoiled DNA was purified for transfection by $CsCl_2$ density gradient centrifugation. Serpin 2A reporter gene plasmids were grown in the presence of ampicillin selection in HB101 *Escherichia coli* and high quality supercoiled DNA was purified by polyethelene glycol precipitation. DNA concentration and covalently closed circular content was determined by ethidium bromide fluorometric analysis in a Sequoia-Turner Model 450 fluorometer.

5. Luciferase and β-galactosidase Assays. Three 10-20 µl aliquots of cell lysates were measured for 20 seconds following the injection of Luciferase Reagent (Luciferase Assay, Promega) by a LUMAT LB9501 luminometer (Berthold Systems Inc.). β-galactosidase assays were performed as described (Sambrook *et al.*, 1989). Final activities are given as luciferase/β-galactosidase values. Because the incubation periods for the β-galactosidase assay varied between the various cell types, the luciferase/ β-galactosidase values are relative only within each transfection experiment but are not relative between the different cell types.

6. Mutagenesis. Site-directed mutagenesis was performed using the Sculptor™ in vitro mutagenesis system (Amersham Corp.). An oligonucleotide containing the desired basepair changes was annealed to a single stranded template and was extended using DNA polymerase. All mutations were confirmed by dideoxy nucleotide sequencing at the DNA laboratory in the department of Biochemistry, University of Alberta.

7. Western Blot Analysis. Cells (3×10^6) were lysed in Triton lysis buffer (0.15 M NaCl, 50 mM Tris[pH 8.0], 1% Triton X-100) and cleared lysates (30 µg each) were separated on a 10% SDS polyacrylamide gel. The proteins were electrotransferred to

an Immobilon-P™ polyvinylidene fluoride membrane (Millipore) and examined for the presence of the granzyme B protein by western blotting. The granzyme B protein was detected using a rabbit anti-mouse α-granzyme B primary antibody, followed by a donkey anti-rabbit antibody conjugated to HRP (Amersham). The immune complexes were visualized using the ECL™ (Amersham Life Science) detection system.

8. Northern Blot Analysis. RNA was prepared by acid guanidinium phenol extraction (Chomczynski and Sacchi, 1987). Total RNA was separated on denaturing formaldehyde agarose gels and transferred onto Hybond-N nylon membranes (Amersham Life Science Inc.) by capillary transfer.

9. DNase1 Hypersensitivity Analysis. Between $1x10^7$ and $1.2x10^7$ $CD8^+$ or $CD4^+$ splenocytes per reaction, unstimulated or stimulated for 3 days (RHFM, 60 U/ml IL-2, 1:500 dilution αCD3, 5 μg/ml concanavalin A, and 1:2000 dilution αCD28) and L cells were washed once in solution 1 (150 mM sucrose, 80 mM KCl, 35 mM HEPES [pH 7.4], 5 mM K_2HPO_4, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$ and 1 mM EDTA). The cells were permeabilized with 0.05% lysolecithin (Sigma Chemical Co.) in solution 2 (150 mM sucrose, 80 mM KCl, 35 mM HEPES [pH 7.4], 5 mM K_2HPO_4, 5 mM $MgCl_2$, 3 mM $CaCl_2$) by immersion for 90 seconds in a 37° C water bath. The cells were washed once with solution 2, and resuspended at $1.2x10^8$ cells/ml in solution 2. Aliquots of 90 μl of permeabilized cells were added to a 10 μl mixture of DNase1 in Solution 2 such that the final concentrations of the reactions were between 0 and 10.0 μg/ml DNase1 or 5 mM EDTA as a control. The reactions were incubated for 5 minutes at 37° C. The reactions were stopped and the cells were lysed by treatment with 10 mM Tris-HCl (pH 8.0), 85 mM NaCl, 10 mM EDTA, 0.5% SDS, and 300 μg/ml proteinase K for 16 hours at 37° C. The genomic DNA was phenol:chloroform extracted 2-3 times and ethanol precipitated. RNA was removed by incubation with 0.1 mg/ml RNAse A at 44° C for 30 minutes, followed by two phenol:chloroform extractions, one chloroform extraction and ethanol precipitation. Each genomic DNA sample (15 μg) was cut with Eco R1 or Avr II and electrophoretically

separated on 1.2% agarose gels. Nucleic acids were transferred to Hybond-N nylon membranes (Amersham Life Science Inc.) by capillary transfer.

10. Electrophoretic Mobility Shift Assays. Nuclear extracts were obtained by the method described in Schreiber *et al.* (1989). Mobility shift assays were performed as in Lin *et al.* (1993) with minor modifications. Briefly, 3-5 µg of nuclear extracts were incubated with 2 µg of poly(dI-dC) and approximately 0.1 to 0.5 ng (10-20,000 cpm) of ^{32}P labeled oligonucleotide in a 15 - 20 µl reaction (containing 12 mM HEPES [pH 7.9], 50 mM KCl, 0.5 mM EDTA, 2.5 mM DTT, and 10% glycerol). Oligonucleotides were end-labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP or annealed and the ends filled in with the Klenow fragment of DNA polymerase I and [α-^{32}P]dCTP. Reactions containing antibody or antiserum were preincubated for 30 minutes at 4° C. The reactions were electrophoretically separated on 5% non-denaturing polyacrylamide gels.

11. In Vitro DNase1 Footprinting. Nuclear extracts were routinely obtained from $1x10^9$ MTL 2.8.2 cells and were prepared as in Ohlsson *et al.* (1986). The footprinting reactions contained approximately 1 ng of end-labeled DNA fragment, 1 µg poly(dI-dC), and 50 or 75 µg of extract in a 50 µl volume buffered by 25 mM HEPES (pH 7.8), 50 mM KCl, 0.05 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF and 5% glycerol. The extract was preincubated with poly(dI-dC) for 20-30 minutes at 4° C, after which the end-labeled fragment was added and incubated for an additional 10-15 minutes at room temperature. The amount of DNase1 added was empirically determined to give an even pattern of cleavage products. Usually 500 ng was used per reaction (2.5 ng for controls) and allowed to digest for 45 sec. DNase 1 digestion was initiated by the addition of $MgCl_2$ and $CaCl_2$ to final concentrations of 5 mM and 1 mM respectively. Digestion was stopped by the addition of 100 µl stop solution (100 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1% Sarkosyl, 10 mM EDTA, 100 µg/ml Proteinase K, and 25 µg/ml calf thymus DNA). DNA was phenol extracted and ethanol precipitated. The fragments were separated on a 7%, 7M urea polyacrylamide sequencing gel and the gels were dried onto filter paper.

12. In Vivo Footprinting by DMS-Piperidine Treatment. Dimethylsulfate (DMS)-Piperidine treatment of cells was performed as described in Mueller *et al.* (1992). Cells were pelleted at 300 x g for 5 minutes. Approximately 1 ml of medium was left behind to resuspend cells. They were then transferred to a microfuge tube and incubated in a 37° C water bath. A 10 µl volume of a 10% DMS/ethanol solution was added to the cells and incubated for 1 minute. The methylation reaction was stopped by transferring cells to 49 ml of ice cold phosphate buffered saline followed by centrifugation at 300 x g for 5 minutes at 4°C. The cell pellet was resuspended in 1-2 ml cold PBS, 49 ml ice cold PBS was added and cells pelleted at 300 x g for 5 minutes at 4°C. The cell pellet was resuspended in 0.3 ml cold PBS and added to 2.7 ml lysis buffer [300 mM NaCl, 50 mM Tris-HCl (pH 8.0), 25 mM EDTA (pH 8.0), 200 µg/ml Proteinase K, 0.2% SDS]. The DNA was purified and piperidine cleavage was performed. A naked DNA control was prepared at the same time.

MTL 2.8.2's were grown on 24x24 cm plates to a final cell density of $2.5\text{-}4x10^7$ cells/plate. The medium was removed and 100 ml of prewarmed RHFM containing 0.1% DMS was added and incubated for 2 minutes. The cells were washed three times with prewarmed PBS. Approximately 8 ml of lysis buffer was layered over the cells and the plates gently swirled for 5 minutes. The lysed cells were scraped off the plates into 50 ml tubes and incubated at 37° C for 5 hrs. DNA was prepared from the lysates and a naked DNA control was processed at the same time. In vitro DMS treatment of naked DNA and subsequent piperidine cleavage of both in vitro and in vivo DMS treated DNA was performed.

13. Ligation Mediated PCR Genomic Footprinting. LMPCR genomic footprinting was performed as detailed in (Mueller *et al.*, 1992). Oligonucleotides used to detect interactions in the noncoding strand of the -243 C11 promoter were:

Primer 1: 5' CCTAGGTCCCAGCGTCAAGAGT 3' (T_m 61.8°C)

Primer 2: 5' GAGAGGAAGAAGGCAGAGGGGGCTCT 3' (T_m 66.3°C)

Primer 3: 5' GAGGAAGAAGGCAGAGGGGGCTCTGTGACC 3' (T_m 69.6°C)

The staggered linker (LMPCR.1) was changed by a single base to raise the T_m. The A residue at position 7 was changed to a G residue. The new T_m was 66°C. LMPCR hybridization temperatures were 63°C, 68°C, and 71°C (for primers 1, 2, and 3 respectively). For the end labeling reaction, 3 cycles of PCR were performed and the final products precipitated in duplicate. The PCR products were run on 7% polyacrylamide, 7M Urea sequencing gels. Fixed and dried gels were exposed on XAR-5 film without an intensifying screen.

14. Oligonucleotides and Probes. The sequences of each oligonucleotide used in EMSA's are as follows:

granzyme B AP-1 agctTCTCTGAGTCATTCa,

granzyme B CBF agctTCTGCTGTGGTTACTTCATa,

granzyme B Ikaros agctTACAACACCCCCACCCCTA,

granzyme B mutant Ikaros GGCTACAACACTCTCACTCCTATGCCCTT

(nucleotides not present in granzyme B are shown in lowercase).

Northern blots were probed with a murine granzyme B cDNA and a human γ-actin cDNA [α-^{32}P]dCTP labeled by random priming. Southern blots obtained in the DNase1 hypersensitivity experiments were probed with a Taq1/Pst1 restriction fragment that extends from -828 to -546 of the granzyme B promoter. A human c-fos gene fragment that extends 80 bp upstream of the transcription start site to 360 bp into intron A was used as a positive control. All hybridizations were performed in 50% formamide hybridization solution at 42° C.

CHAPTER 3[1]

5' FLANKING SEQUENCES IN THE REGULATION OF GRANZYME B TRANSCRIPTION

INTRODUCTION

The body's major defense against viral infections is T cell-mediated cytotoxicity whereby specialized cells seek and destroy any cell that can potentially harm the organism. Unfortunately, this line of immunity is a double edged sword in that these cells can sometimes mount an attack on healthy cells and cause autoimmune diseases and they are primarily responsible for organ and tissue transplant rejection. Whether the response is appropriate or not, resting T lymphocytes become activated in response to foreign antigen recognition and one approach to understanding the events that occur is to study the specialized set of genes that are induced during the acquisition of killing potential. These include perforin and cytotoxic serine proteinases (granzymes) that are major components of the killing machinery.

Granzymes are implicated in granule-mediated target cell death because their expression is closely correlated with killing activity (Prendergast *et al.*, 1992b) and by their localization in cytolytic granules that are exocytosed from CTL and natural killer (NK) cells upon interaction with target cell membranes (Henkart, 1985; Redmond *et al.*, 1987). Granzyme B is a member of the cytotoxic serine proteinase gene family that is clustered on mouse chromosome 14 (Crosby *et al.*, 1990). The granzyme genes provide an excellent system in which to study cell-specific gene induction as their expression is restricted to activated T lymphocytes and NK cells. Studies of the human and murine granzyme B proximal promoters reveal that they share several highly conserved sequences. These

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[1] A version of this chapter has been published: Babichuk, C.K., B.L. Duggan, and R.C. Bleackley. 1996. *J. Biol. Chem.* **271**:16485.

include T cell-specific transcription factor binding sites, identified within the human promoter, such as Ikaros and core binding factor (CBF) (Haddad *et al.*, 1993, Kamachi *et al.*, 1990) as well as binding sites for the ubiquitous transcription factors AP1 and the cyclic-AMP response element binding factor. These sequences have been shown to be sufficient to induce reporter gene expression by transient transfection analysis in immortalized T cell lines in which many of these transcription factors are constitutively active (Frégeau and Bleackley, 1991; Hanson *et al.*, 1990; Hanson *et al.*, 1993).

The events that take place at the endogenous granzyme B locus as resting lymphocytes make the transition to activated killers are of primary interest to those desiring an understanding of T cell-specific gene induction. The regulatory sequences that comprise the granzyme B promoter serve as docking sites for both ubiquitous and lymphoid-specific transcription factors that are responsible for the induction and maintenance of the actively transcribed gene. The opportunity allowing these proteins to converge on the promoter is governed not only by the presence of the factors but also by their accessibility to the DNA. The studies described in this chapter identify and localize the important 5' regulatory sequences involved in granzyme B transcription in activated cytotoxic T cells.

$CD8^+$ T cells were isolated from primary splenocyte cultures, which can be experimentally monitored from the earliest stages of induction throughout fully activated transcription. A major portion of our research investigated the resting vs. the activated state. We have developed a method for the transfection of reporter gene plasmids into primary murine lymphocytes and showed that the minimal granzyme B promoter is able to induce significant levels of luciferase activity in activated $CD8^+$ T cells. Electrophoretic mobility shift analysis was used to examine the DNA binding activities of transcription factors before and after $CD8^+$ T cell activation. We have precisely established the sequences involved in transcription factor binding by in vitro footprinting studies using nuclear extracts derived from a cytotoxic T cell clone. DNase1 hypersensitivity analysis identified potentially important regulatory regions in the endogenous granzyme B promoter

in $CD8^+$ cells. Finally, we were able to observe these protein/DNA interactions in the endogenous promoter by in vivo footprinting analysis in resting and activated $CD8^+$ T cells using the dimethylsulfate (DMS)/LMPCR genomic footprinting method. Together these results have enabled us to probe the status of the endogenous gene before and after T cell activation and in a physiologically relevant system.

RESULTS

Granzyme B RNA is CTL-Specific

To determine the expression pattern of the granzyme B RNA in the various cell types utilized in our experiments, we prepared total RNA and probed with a granzyme B cDNA. Figure 3-1 depicts the relative mRNA levels that were produced from two murine cytotoxic T cell lines, the IL-2-dependent MTL 2.8.2 line, the IL-2 and alloantigen-dependent CTL21.9 (Type1) line, and stimulated $CD8^+$ splenocytes. Also included were EL4, a murine thymoma T cell line, L cell fibroblasts, and a granzyme-independent cytolytic hybridoma PMM-1 (Kaufman *et al.*, 1982). The granzyme B mRNA was expressed at high levels in $CD8^+$ splenocytes and our cytotoxic T cell clones but was completely absent in EL4, PMM-1, or L cells. We were not able to induce further the mRNA levels in Type1 T cells or induce transcription in EL4 cells by stimulation with PMA/ionomycin, αCD3, or concanavalin A (data not shown).

The granzyme B mRNA was inducible, however, in primary splenocytes stimulated in the presence of IL-2, αCD3, and concanavalin A. We examined this induction in a population of primary lymphocytes by Northern blot analysis after one, two, and three days following stimulation. On day three, lymphocytes were isolated by immunomagnetic separation and total RNA was prepared for analysis. Figure 3-2 depicts the induction profile of the granzyme B message in the whole splenocyte population and of the mRNA in the $CD8^+$ and $CD4^+/CD8^+$ depleted cells. Granzyme B mRNA appeared on day one, was very high by day three, and was limited to the $CD8^+$ T cell fraction. Together, this

Figure 3-1

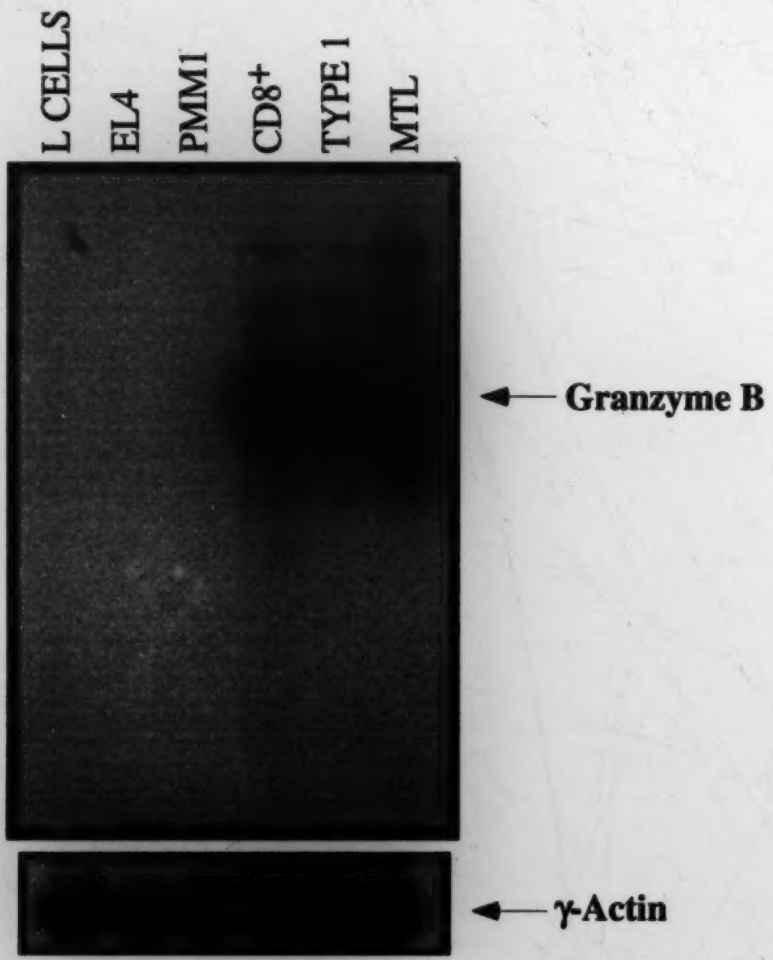


Figure 3-1. Northern blot analysis of the granzyme B mRNA in various cell types.

Total RNA was collected from L cells, EL4, PMM1, $CD8^+$ (stimulated for 3 days with IL-2, αCD3 and Con A and isolated by passage over a CD8 immunocolumn), Type1, and MTL 2.8.2 cells and 5 μg of each was separated on a 0.9% denaturing formaldehyde/agarose gel. The resulting blot was probed with a murine granzyme B cDNA and a human γ-actin cDNA.

Figure 3-2

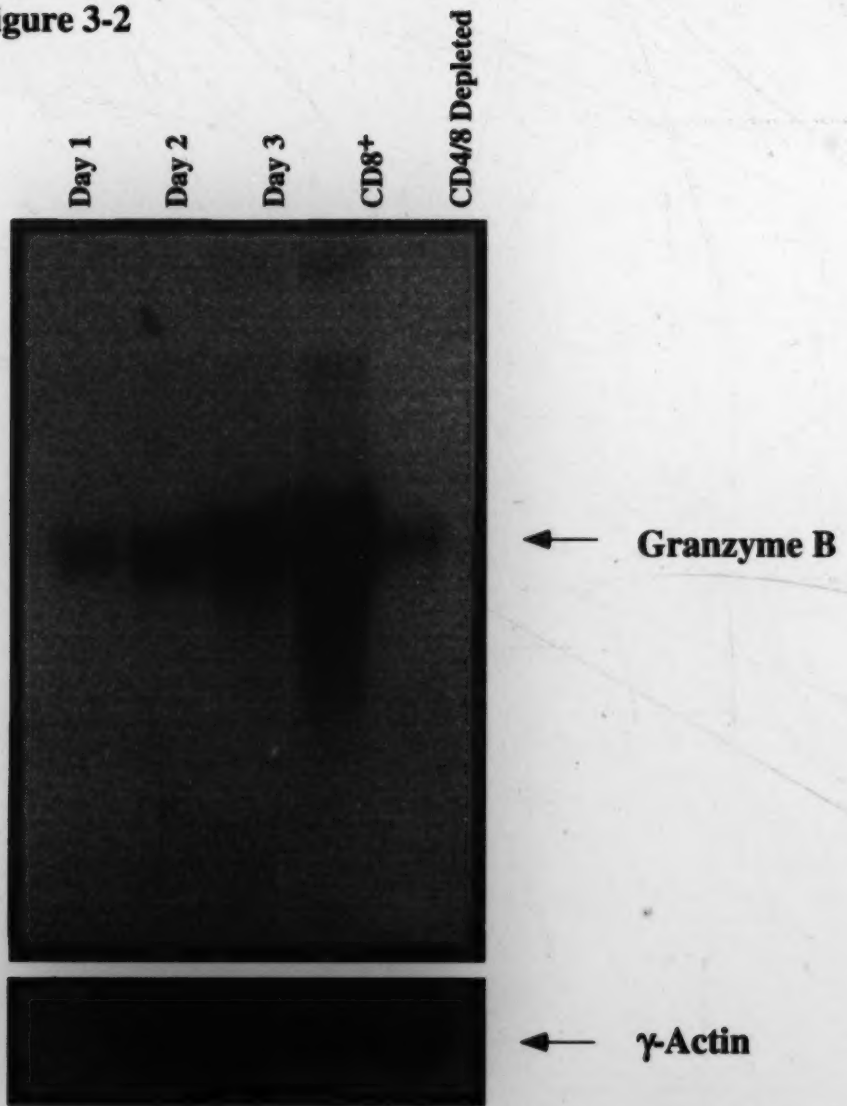


Figure 3-2. Northern blot analysis of granzyme B mRNA accumulation in stimulated whole and CD8+ (day 3) primary splenocytes.

Total cellular RNA was collected from whole splenocytes following 1, 2, or 3 days of stimulation with IL-2, αCD3, and Con A and from the day three $CD8^+$ (isolated by positive immunomagnetic separation), and $CD4^+/8^+$ depleted subpopulations. RNA (5 μg) was separated on a 0.9% denaturing formaldehyde/agarose gel and the resultant Northern blot was probed with a murine granzyme B cDNA and a human γ-actin cDNA.

Northern blot data indicates that this gene is only efficiently transcribed, or the mRNA is only sufficiently stable, in cytotoxic T cells.

A 243 bp Promoter Fragment Confers High Levels of Reporter Gene Activity in T Cells and L Cells in Transient Transfection Analysis

To define important transcriptional regulatory elements in the granzyme B promoter, a series of deletion fragments was constructed and inserted upstream of a promoterless luciferase gene. These constructs were then transfected into a variety of cell types and the relative levels of reporter gene expression examined. Two promoter fragments, one that extends 243 bp and another that extends 828 bp upstream from the transcription start site consistently produced the highest levels of luciferase activity in Type1 CTL cells (Fig. 3-3A). The Rous Sarcoma viral promoter was typically as active as the 828 bp granzyme B (C11 gene clone) promoter in T cells and background levels of luciferase expression were observed from the parental p19LUC plasmid. Larger 5' flanking sequences were examined (up to 5 kb) and were much less effective in activating luciferase gene expression than the 828 bp or the 243 bp fragments in T cells and non-T cells (data not shown). We decided to focus our studies on the smaller, but highly active, 243 bp fragment.

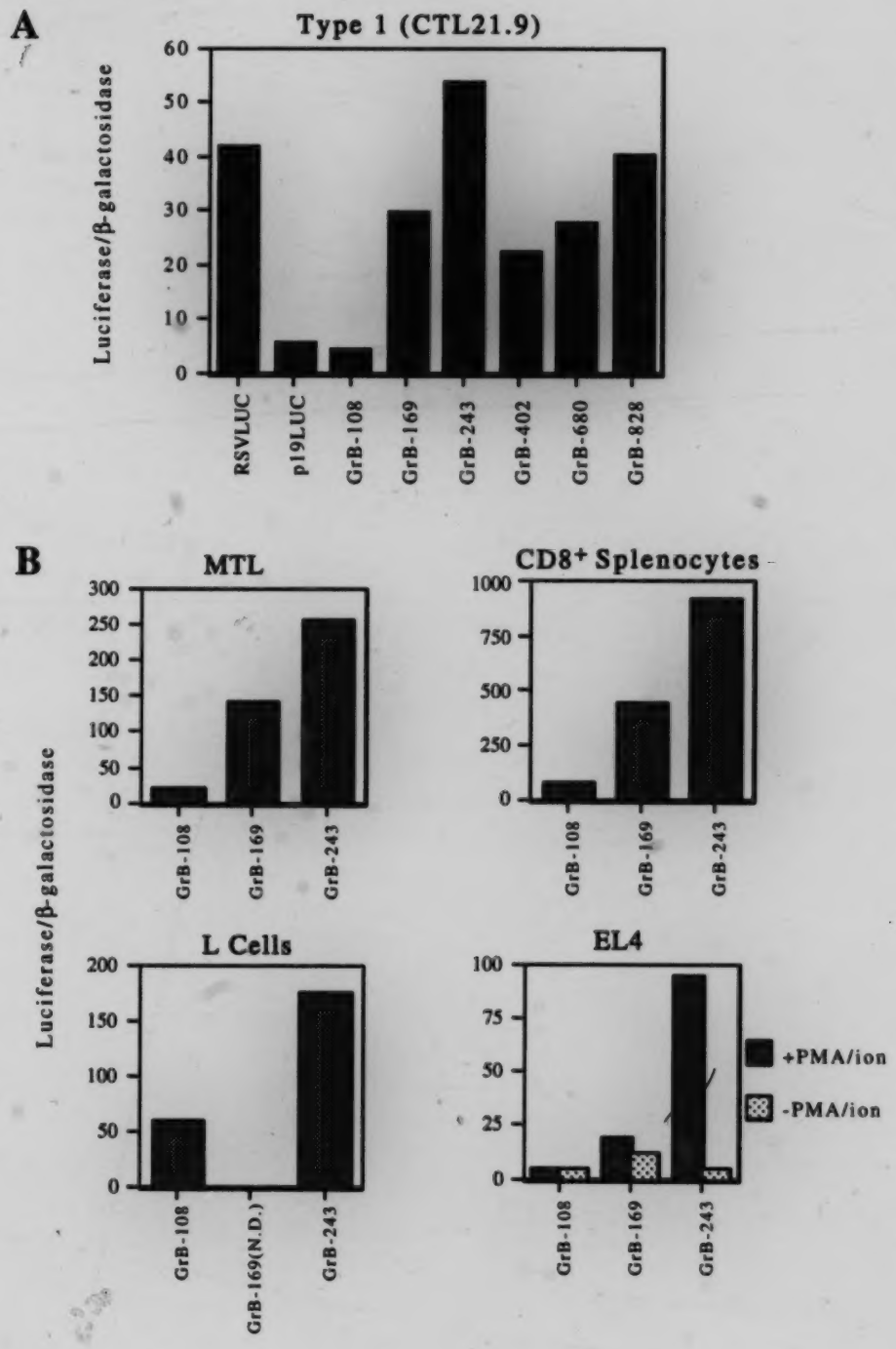
Fragments extending 108 bp, 169 bp, and 243 bp upstream of the transcription start site were transfected into MTL 2.8.2, EL4, L cells, and whole splenocytes. Splenocytes were activated by stimulation with IL-2, αCD3, and concanavalin A for approximately 20 hours prior to transfection, and were then re-exposed to stimulus for another two days. The immunomagnetic separation of the $CD8^+$ fraction was performed prior to harvest on day three post initial activation. Significant increases in luciferase activity were observed in all cell types, except EL4, as the 3' CBF and AP1 binding sites (contained within -169) and the 5' CBF and Ikaros binding sites (contained within -243, see below) were included in the constructs (Fig. 3-3B). We did observe, however, a low but significant level of luciferase expression in EL4 cells upon stimulation with PMA/ionomycin. Apparently, two

Figure 3-3 Transient transfection analysis of granzyme B (C11) promoter deletion fragments in T cells and L cells.

A. The granzyme B promoter/luciferase deletion series was transfected into a Type1 (CTL21.9) cytotoxic T cell line and relative expression values for one experiment are shown as a luciferase/β-galactosidase activity ratio.

B. Various promoter/luciferase constructs were transfected into MTL 2.8.2, stimulated $CD8^+$ splenocytes (immunomagnetically separated), L cells, and EL4 helper cells (resting or activated for 20 hours with 10 ng/ml PMA and 4 μM ionomycin). The relative expression values are shown as a luciferase/β-galactosidase activity.

Figure 3-3


A
Type 1 (CTL21.9)
Luciferase/β-galactosidase
60
50
40
30
20
10
0
RSVLUC
p19LUC
GrB-108
GrB-169
GrB-243
GrB-402
GrB-680
GrB-828
B
MTL
CD8+ Splenocytes
L Cells
EL4
Luciferase/β-galactosidase
300
250
200
150
100
50
0
1000
750
500
250
200
150
100
50
0
100
75
50
25
0
GrB-108
GrB-169
GrB-243
GrB-169(N.D.)
+PMA/ion
-PMA/ion

major elements, one between -108 and -169, and another between -169 and -243, seem to be very important for the high levels of reporter gene expression observed from the -243 fragment and for the inducibility by PMA/ionomycin in EL4 cells. These transfection studies indicate that the minimal granzyme B promoter confers high levels of expression in transient assays but is not necessarily restricted to T cells.

The Granzyme B -243 Promoter Contains Binding Sites for Four Known Transcription Factors

Within the -243 promoter fragment there exist consensus sequence binding sites for the transcription factors AP1, core binding factor (CBF), Ikaros, and the cyclic AMP-responsive element (CRE). These sites are located at approximately -200 (Ikaros), -180/ -126 (CBF), -150 (AP1) and at -90 (CRE) nucleotides relative to the transcription start site (Fig. 3-4).

Nuclear extracts were prepared from MTL 2.8.2 T cells, L cells, and PMA/ionomycin stimulated and unstimulated EL4 cells, and incubated with oligonucleotides containing the granzyme B AP1 and CBF sequence elements. Electrophoretic mobility shift assays (EMSA) showed that both binding sites formed specific complexes with nuclear proteins present in all of these cells (Fig. 3-5A). These results show that the AP1 and CBF regulatory factors are present in the nuclei of cells that both express and do not express granzyme B.

Mobility shift assays were then performed using nuclear extracts from purified $CD8^+$ murine splenocytes to compare complexes in resting and activated cells. Figure 3-5B shows that the granzyme B AP1 oligonucleotide formed a complex with nuclear extracts from stimulated $CD8^+$ cells whereas this complex was absent in resting splenocytes. It has been previously observed that c-fos mRNA is absent in resting T cells (Jain *et al.*, 1992). A supershift was observed in activated cells with a c-Fos antibody although the c-Jun antibody used in this assay had a negligible effect on the complex. These results indicate

Figure 3-4

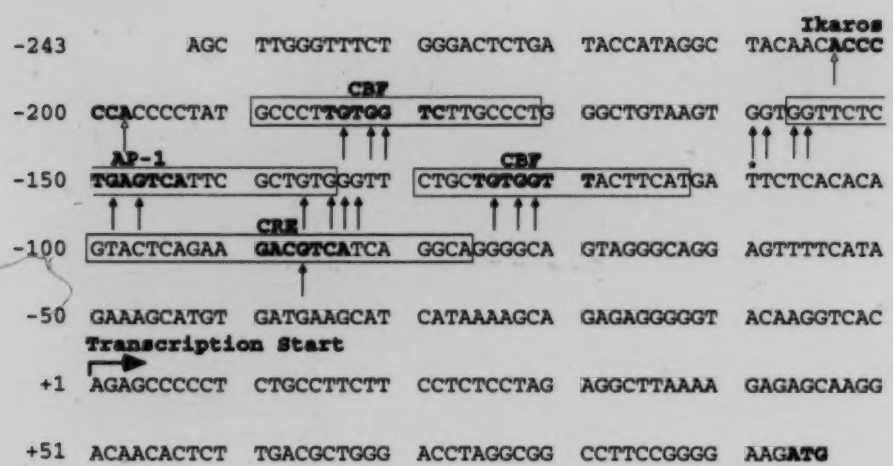


Figure 3-4 The nucleotide sequence of the granzyme B proximal promoter.

Major potential transcription factor binding sites are in boldface and the transcription factor designations are shown above the sequences (AP-1: activator protein-1, CBF: core binding factor, and CRE: cyclic-AMP responsive element). In vitro footprints are shown in boxes. Upward arrows denote protected G residues of in vivo footprints and an asterisk denotes the hypersensitive residue. The open arrows indicate hypersensitive residues found only in MTL 2.8.2. The transcription start site is indicated with an arrow and the translation initiation codon (ATG) is indicated in boldface.

that in resting splenocytes the AP1 complex is either absent or does not bind to DNA and activation through the T cell receptor is required for effective DNA binding activity.

The granzyme B CBF oligonucleotide formed two complexes in $CD8^+$ lymphocytes (Fig. 3-5C). In nuclear extracts obtained from resting splenocytes, a weak, indistinct complex was formed with the CBF oligonucleotide. Upon stimulation of the cells for 45 hours with αCD3, a clear, slower mobility complex was observed. The complex was inhibited when the granzyme B CBF oligonucleotide was incubated with nuclear extracts in the presence of a 50 or 200 molar excess of unlabeled granzyme B CBF oligonucleotide. An interesting observation was made when antisera to either PEBP2 αA (CBF α-subunit) or PEBP2β (CBF β-subunit) was added to the reaction. The anti-αA serum did not appear to affect the major complex, however, the anti-β serum was capable of disrupting the complex. The αA antiserum cross reacts with all three of the known α subunits, (personal communication, Y. Ito) including αB and αC, which are expressed at high levels in T cells. We may be observing an as yet unknown variation of the α subunit or an entirely different protein that is capable of binding to the CBF/PEBP2 binding site and interacting with the β subunit.

It has been previously determined that the Ikaros gene gives rise to a lymphoid-restricted family of functionally distinct transcription factor proteins which are involved throughout lymphocyte development (Georgopoulos, *et al.*, 1994; Molnar and Georgopoulos 1994). The Ikaros element in granzyme B formed a specific complex that was present in both unstimulated and αCD3 stimulated $CD8^+$ splenocytes (Fig. 3-5D). This complex was competed off with an excess of the granzyme B binding site whereas competition with a mutant Ikaros binding site did not affect the complex. To confirm the binding of regulatory factors along the granzyme B promoter we performed in vitro DNase 1 footprinting. When the granzyme B -243 promoter fragment was incubated with MTL 2.8.2 nuclear extracts and treated with DNase1, four areas of protection from DNase1 digestion were evident (Fig. 3-6). Two distinct footprints were visible over the AP1 and

Figure 3-5 Nuclear extracts from T cells and non-T cells form complexes with oligonucleotide probes containing the granzyme B AP1, CBF, and Ikaros binding sites.

A. Nuclear extracts prepared from MTL 2.8.2, EL4 (unstimulated or stimulated with 10 ng/ml PMA and 4 μM ionomycin), and L cells were incubated with the granzyme B AP1 or CBF oligonucleotides and subject to electrophoresis on a non-denaturing polyacrylamide gel.

B. The radiolabeled granzyme B AP1 oligonucleotide was incubated with 2 μg of nuclear protein extracts prepared from unstimulated and αCD3 stimulated (two days in the presence of IL-2) $CD8^+$ splenocytes. Competition experiments were performed in the presence of 50 and 200 fold molar excess of unlabelled probe. For supershift binding assays, 0.5 μg of anti-c-fos or anti-c-jun antibody were added.

C. The same nuclear extracts as in (B) were incubated with the radiolabeled granzyme B CBF oligonucleotide. 3 μl of the αA or β antiserum was added as indicated. Competition experiments were performed in the presence of 50 and 200 fold molar excess of unlabelled probe.

D. The same extracts used in (B) and (C) were incubated with the granzyme B Ikaros oligonucleotide. Competition experiments were performed in the presence of 50 and 200 fold molar excess of unlabelled probe.

These mobility shift experiments were performed by Brenda L. Duggan.

Figure 3-5

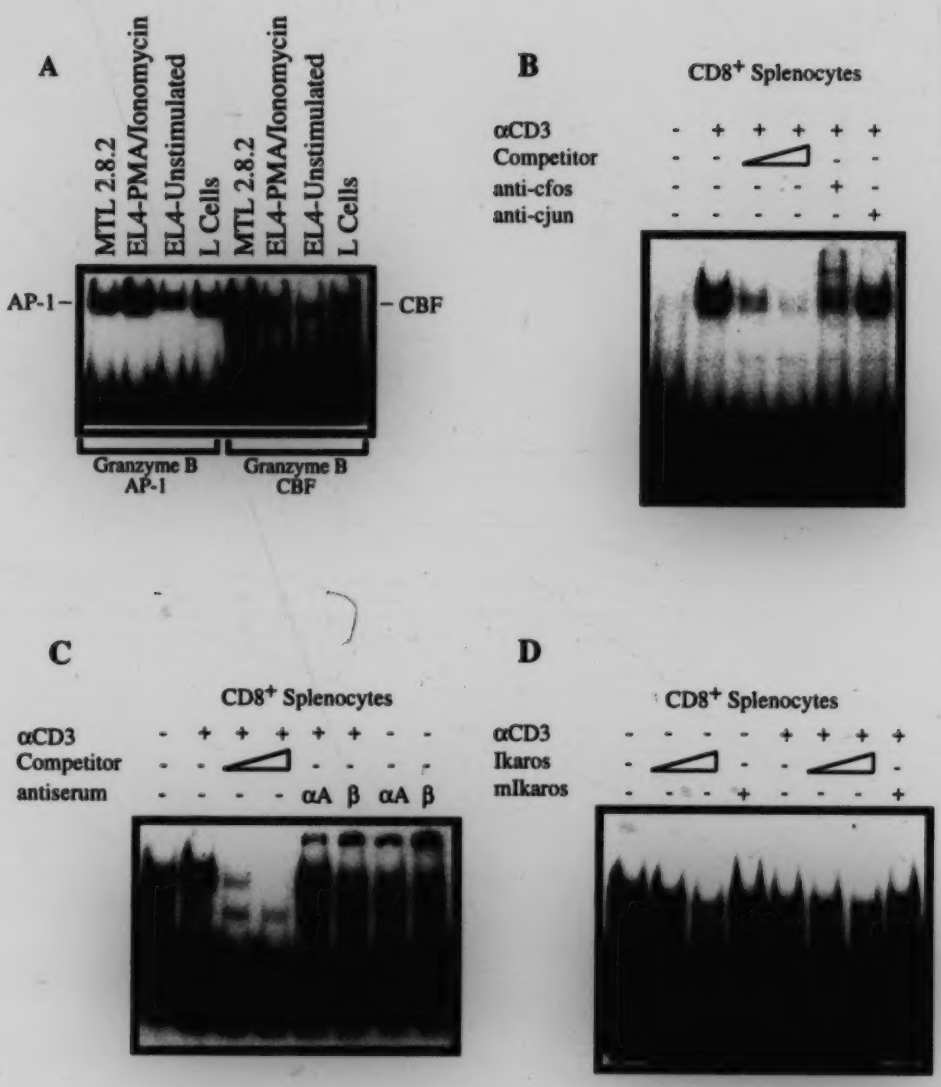
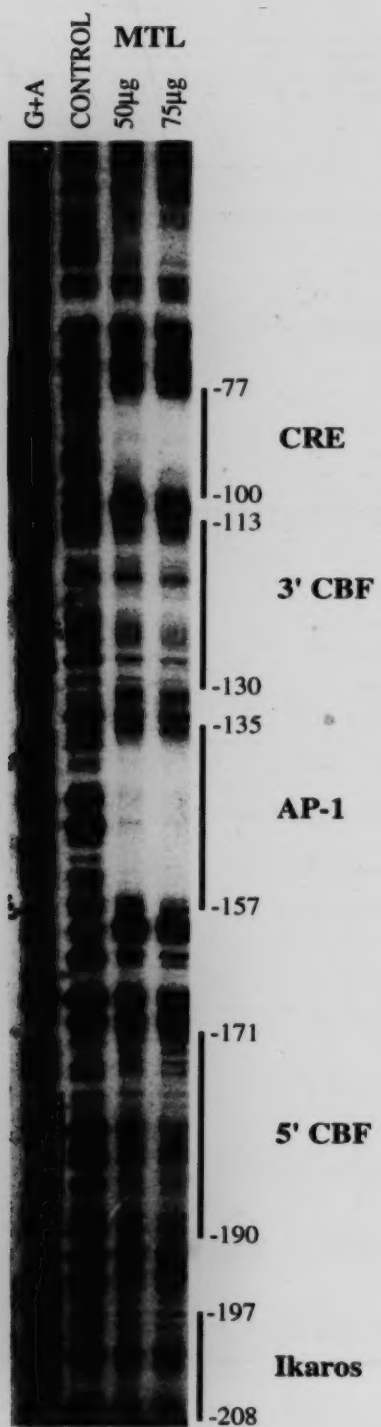

A
MTL 2.8.2
EL4-PMA/Ionomycin
EL4-Unstimulated
L Cells
MTL 2.8.2
EL4-PMA/Ionomycin
EL4-Unstimulated
L Cells
AP-1
CBF
Granzyme B AP-1
Granzyme B CBF
B
CD8+ Splenocytes
αCD3
Competitor
anti-cfos
anti-cjun
C
CD8+ Splenocytes
αCD3
Competitor
antiserum
αA β αA β
D
CD8+ Splenocytes
αCD3
Ikaros
mIkaros

Figure 3-6 In vitro DNase1 footprint analysis with MTL nuclear extracts shows clear footprints at the AP1, CBF, and CRE elements.

MTL 2.8.2 nuclear extracts (50 or 75 μg) were incubated with an end labeled granzyme B promoter fragment and subject to digestion with DNase1. The cleavage products were separated on a 7%, 7M Urea polyacrylamide gel. A control reaction containing the cleavage products of naked DNA and a Maxam-Gilbert G+A cleavage ladder were included in the two left lanes. A less prominent footprint over the Ikaros element is also observed. This work was performed by Brenda L. Duggan.

Figure 3-6


G+A
CONTROL
MTL
50µg
75µg
-77
CRE
-100
-113
3' CBF
-130
-135
AP-1
-157
-171
5' CBF
-190
-197
Ikaros
-208

the CRE sequence elements. Less distinct footprints were detected over the two CBF elements and the Ikaros element. Together, this series of assays reveal that there are both activation-dependent and ubiquitous transcription factors binding to the granzyme B promoter in $CD8^+$ splenocytes.

Activated $CD8^+$ Splenocytes Possess a Strong DNase1 Hypersensitive Site That is Absent in Resting $CD8^+$ Cells and L Cells

Permeabilization of cell membranes with lysolecithin allows DNase1 to penetrate into living cells and cleave exposed regions of DNA within intact nuclei. Regions of chromosomal DNA that are accessible to, or are bound by, transcription factors tend to be hypersensitive to DNase1 digestion. DNase1 hypersensitive sites can be visualized on Southern blots with probes designed to specifically end-label a restriction fragment that contains the site of interest. The genomic locus containing the granzyme B gene has been mapped by Corrinne Lobe, Eric Atkinson, and Nancy Ehrman (Fig. 3-7). The granzyme B gene is contained within a 4.5 kb Eco RI fragment whose 5' end is 961 bp upstream of the transcription start site. A restriction fragment probe that extends from -828 to -546 relative to the transcription start site and does not contain repetitive sequence elements was used to end label this fragment.

Resting $CD8^+$ lymphocytes were isolated by passage of whole splenocytes over a CD8 immunocolumn designed to retain B cells and the opposing T cell subset while enriching for the cell type of interest in the flowthrough. The cells were collected, permeabilized with lysolecithin, and treated with increasing amounts of DNase1. Genomic DNA was isolated, cut with Eco RI, separated by electrophoresis on an agarose gel, blotted onto a nylon membrane, and probed. It was observed that the 4.5 kb Eco R1 fragment gradually decreased in intensity with higher concentrations of DNase1 (Fig. 3-8A). No specific sub-bands appeared that would indicate a hypersensitive region.

Figure 3-7

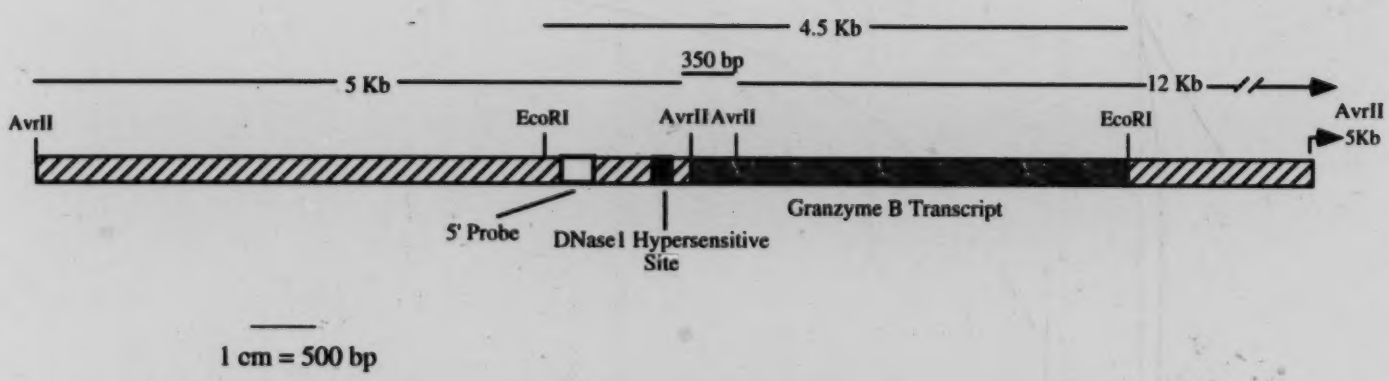


Figure 3-7. The granzyme B genomic locus map and the location of the 5' promoter probe.

A map of the Eco RI and Avr II sites in the granzyme B genomic locus is shown. The transcribed region is shown in gray. The sizes of the various restriction enzyme fragments obtained with Eco RI and Avr II are indicated above the locus and are delineated by lines. The granzyme B 5' probe that end labels the 4.5 Kb Eco RI fragment is depicted as a white box and the hypersensitive region is shown in black.

In activated T cells, however, an area just proximal to the transcription start site was highly sensitive to DNase1 cleavage. Whole splenocytes were passed over a large T cell column and activated for three days (IL-2, αCD3, concanavalin A, and αCD28). The $CD4^+$ T cells were removed by immunomagnetic separation and the $CD8^+$ cells were prepared for DNase1 hypersensitivity analysis as above. As seen on the resultant Southern blot, the 4.5 kb Eco R1 fragment diminished with higher concentrations of DNase1 and a 750 to 900 bp sub-band appeared (Fig. 3-8B). The boundaries of this roughly 150 bp hypersensitive region directly correspond to the sequences which contain the AP1, CBF, Ikaros, and CRE transcription factor binding sites. We were not able to detect a corresponding hypersensitive site in L cell nuclei (Fig. 3-8C). Thus, even though there are nuclear factors in L cells that are capable of binding to granzyme B promoter sequences in vitro, they are not binding to the endogenous, chromosomal DNA. As a positive control, the constitutively active, c-fos gene promoter was probed in resting splenocytes and L cells. Together, this series of experiments indicates that the granzyme B promoter undergoes a structural modification upon T cell activation that allows transcription factors access to the locus and this phenomenon is cell type-specific and activation-dependent.

To determine if any other DNase1 hypersensitive regions exist in activated $CD8^+$ cells outside of the 4.5 kb Eco RI fragment the same DNase1 digested DNA as above was cut with the restriction enzyme Avr II. Avr II digestion gives rise to three genomic bands that can be detected with granzyme B probes. The most 5' band is 5 kb in length and is detected with the same proximal promoter probe as above. The next band is 350 bp in length and encompasses part of the 5' untranslated region, exon 1, and part of intron 1. The 3' most band is approximately 12 kb in length and contains the rest of the transcribed sequences and 9 kb of 3' flanking sequences. The last two bands would hybridize with a granzyme B cDNA probe.

Figure 3-8 DNase1 hypersensitivity analysis in resting and activated CD8$^+$ splenocytes that the granzyme B promoter is hypersensitive to DNase1 digestion in activated T lymphocytes.

Lysolecithin permeabilized cells were treated with between 0 and 10.0 µg/ml of DNase1. Genomic DNA was cut with Eco R1, separated on 1.2% agarose gels (15 µg/lane), and transferred to nylon membranes. The blots were probed with a 279 bp 5' granzyme B probe that indirectly end-labels a 4.4 kb Eco R1 fragment whose 5' end is 961 bp upstream of the first transcribed nucleotide and a c-fos promoter probe as a positive control.

A. The Southern blot of CD8$^+$ unstimulated splenocytes does not indicate hypersensitive regions (6 day exposure; longer exposures of up to 2 weeks failed to reveal any hypersensitive sites). Resting CD8$^+$ lymphocytes were isolated from whole primary splenocytes by passage over a CD8 immunocolumn. CD8$^+$ cells comprised 71% of the enriched population and CD4$^+$ accounted for <1.2%.

B. Hypersensitive site formation in CD8$^+$ splenocytes stimulated for three days with IL-2, αCD3, concanavalin A, and αCD28 (2 week exposure). Stimulated CD8$^+$ lymphocytes from a culture of mixed T cells (isolated by passage through a T cell immunocolumn) were depleted of CD4$^+$ cells by immunomagnetic separation with Dynabeads™. The population is >70% pure. Size standards and the hypersensitive region are indicated to the right of the figure.

C. The granzyme B locus is relatively insensitive to cleavage by DNase1 in L cell fibroblasts (6 day exposure).

Figure 3-8

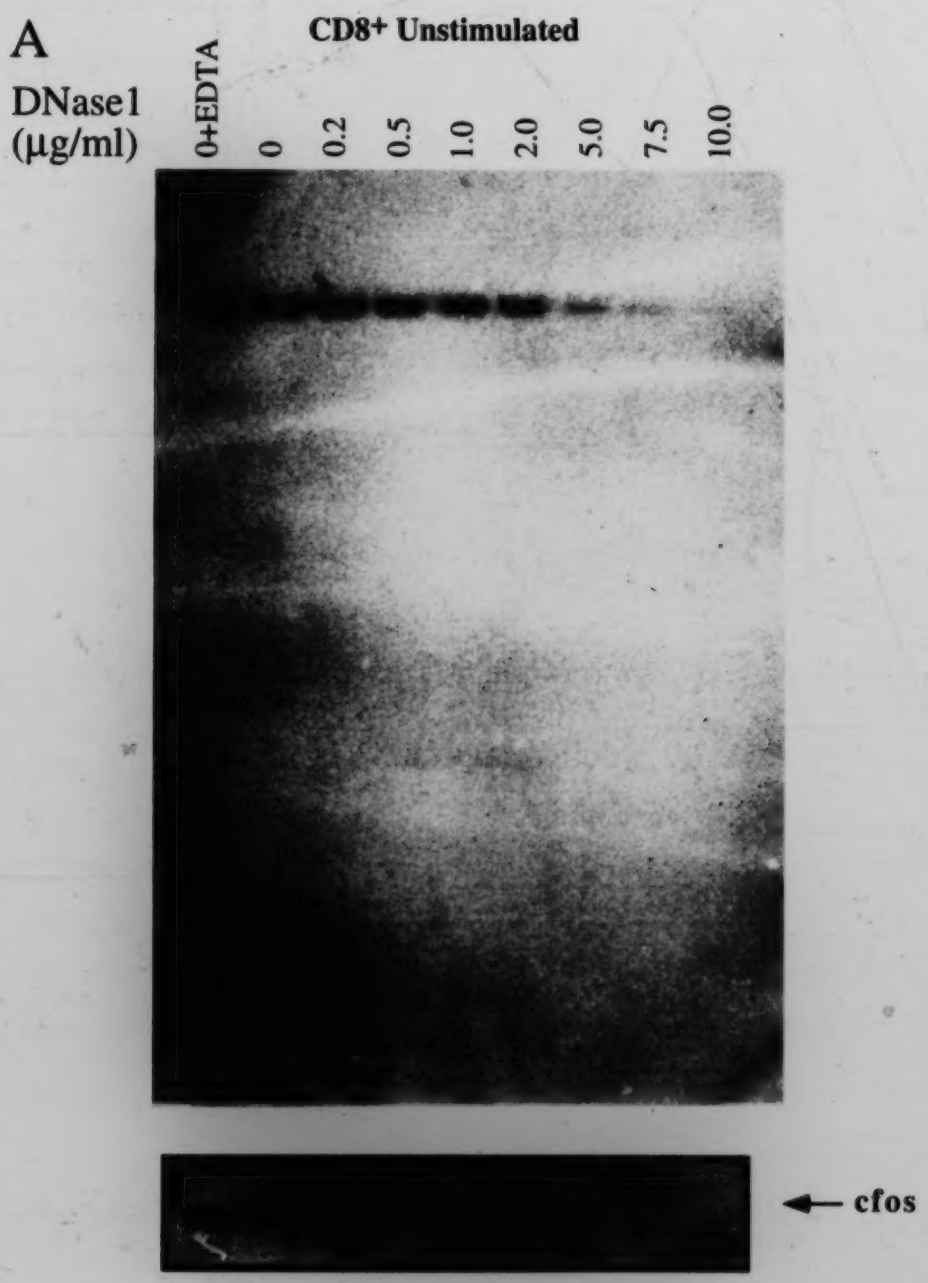

A
CD8+ Unstimulated
DNase1
(µg/ml)
0+EDTA
0
0.2
0.5
1.0
2.0
5.0
7.5
10.0
cfos

Figure 3-8

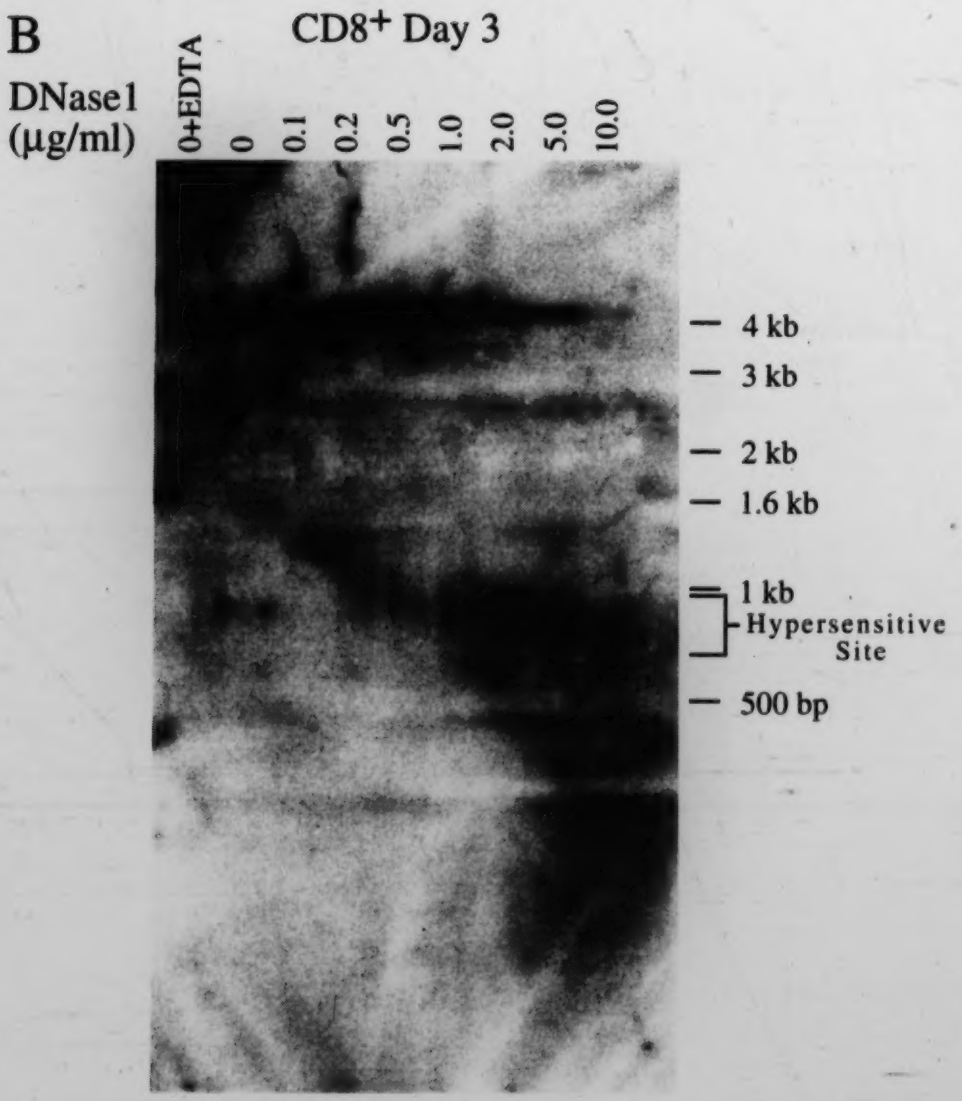
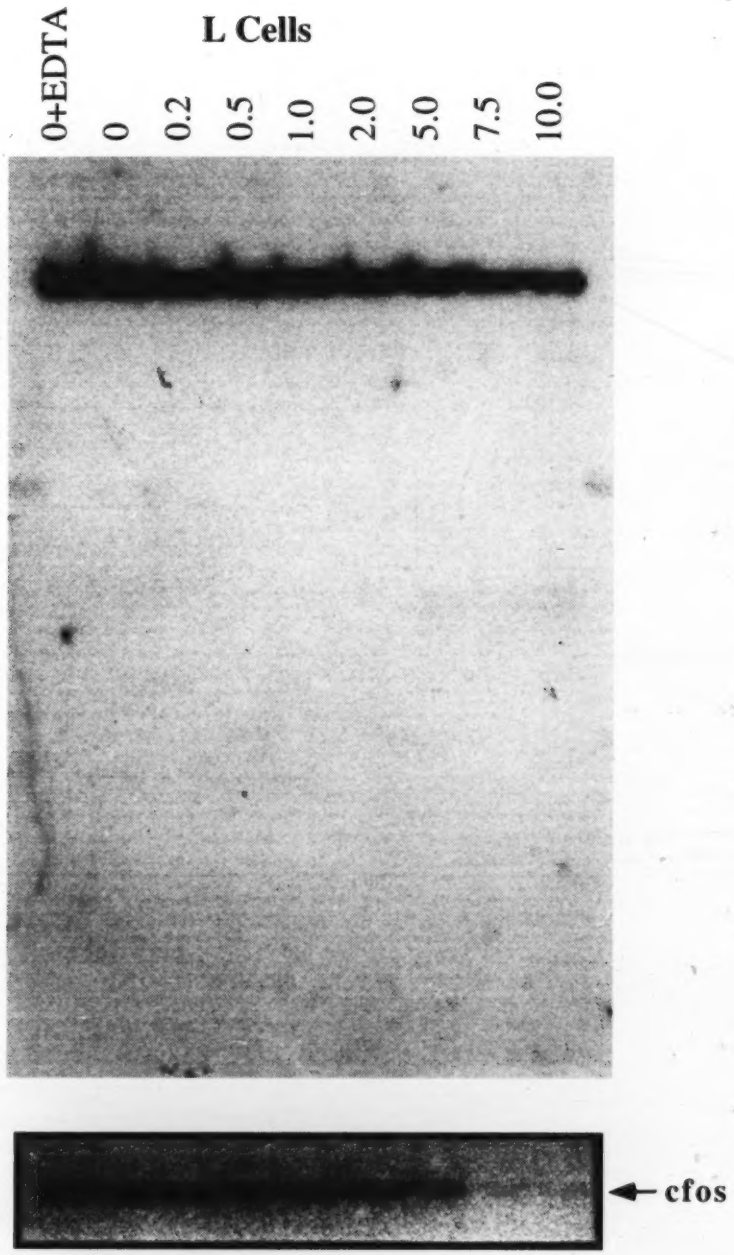

B
CD8+ Day 3
DNase1
(µg/ml)
0+EDTA
0
0.1
0.2
0.5
1.0
2.0
5.0
10.0
4 kb
3 kb
2 kb
1.6 kb
1 kb
Hypersensitive
Site
500 bp

Figure 3-8

C



The Southern blot obtained with Avr II digested DNA was probed with the granzyme B 5' probe and the 5 kb bands gradually faded with increasing concetrations of DNase1 (Fig. 3-9A). A sub-band of 4.2 kb appeared with higher concentrations of DNase1 and indicates a hypersensitive site in the proximal promoter region, corresponding to the one identified above. No other sub-bands appeared, indicating that no other hypersensitive regions exist between the proximal promoter and 5 kb upstream of the transcription start site. The same Southern blot was then probed with a granzyme B cDNA. The 350 bp Avr II fragment was not visible on this Southern blot due to its small size and the low resolution limits of the gel. The 12 kb Avr II fragment was visible and gradually faded with increasing concentrations of DNase1 (Fig. 3-9B). The bands began to fade with 2 U/ml DNase1 and were almost completely digested with 10 U/ml. This digestion pattern is very similar to pattern detected with the granzyme B 5' probe. This suggests that the 3' end of the gene is as sensitive to overall DNase1 digestion as the 5' end. Again, no specific sub-bands appeared that would be indicative of a hypersensitive area within or 3' of the granzyme B gene. The 3.4 kb band that is present in all lanes is likely a granzyme B locus containing fragment that arose due to contaminating nuclease activity in the digestion reaction. Together these results establish that the proximal promoter is the only site in the genomic granzyme B locus that is hypersensitive to nucleases, and is readily accessible to transcription factors, in activated primary $CD8^+$ lymphocytes.

Granzyme B Activation Correlates With Binding of Regulatory Proteins In Vivo

In vivo footprinting is a powerful assay that permits direct detection of protein/DNA interactions within the intact appropriate cell type. Moreover, this method can discriminate between accessible and inaccessible protein binding sites in the chromatin of living cells. In vivo footprint analysis in MTL 2.8.2 cells showed that in intact cells there exist several regions in the granzyme B promoter that were protected from dimethylsulfate (DMS)

Figure 3-9 Further DNase1 hypersensitivity analysis in activated CD8$^+$ T lymphocytes did not reveal other hypersensitive sites.

The DNase1 digested genomic DNA from three day stimulated CD8$^+$ lymphocytes was digested with Avr II and separated on 1.2% agarose gels (10 µg/lane).

A. The Southern blot was probed with the granzyme B 5' fragment (2 week exposure). Size standards and the hypersensitive region are indicated to the right of the figure.

B. The same Southern blot was probed with a granzyme B cDNA probe (2 week exposure). Size standards are indicated to the right of the figure.

Figure 3-9

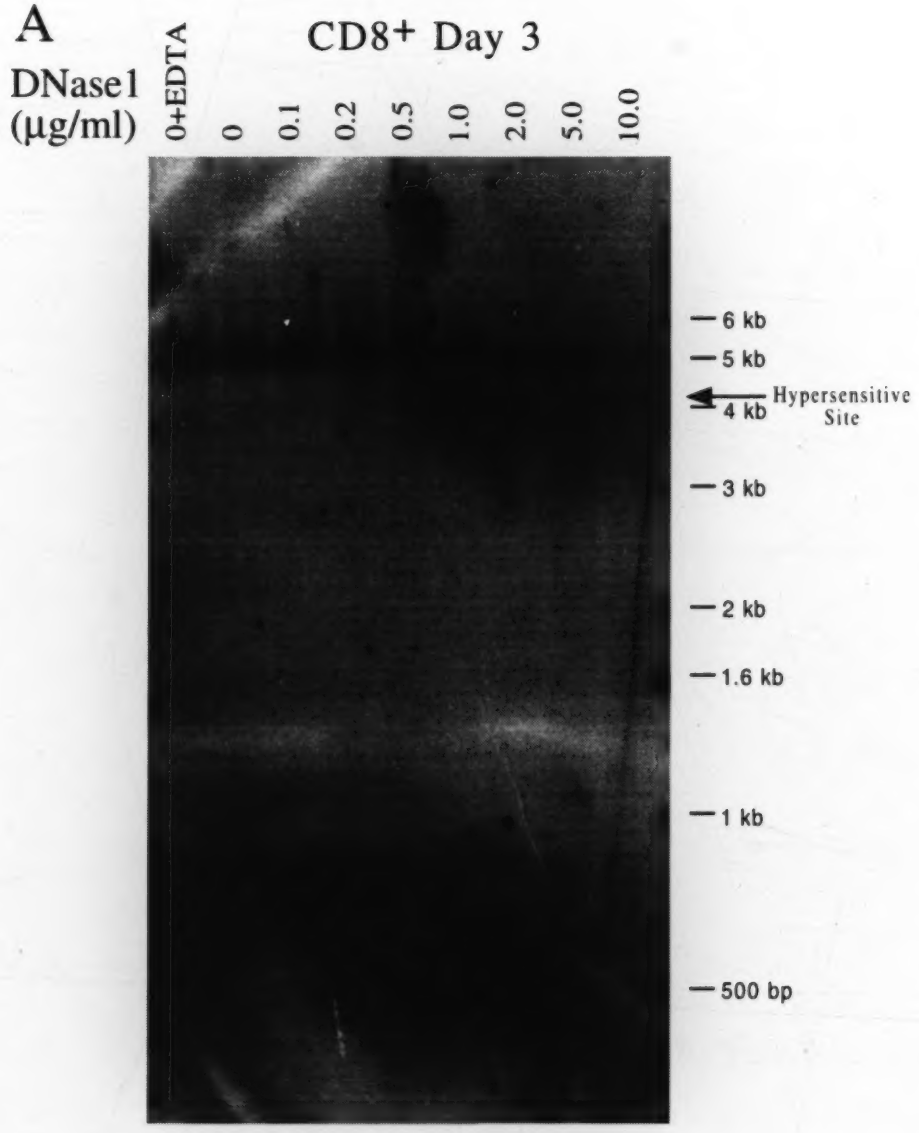
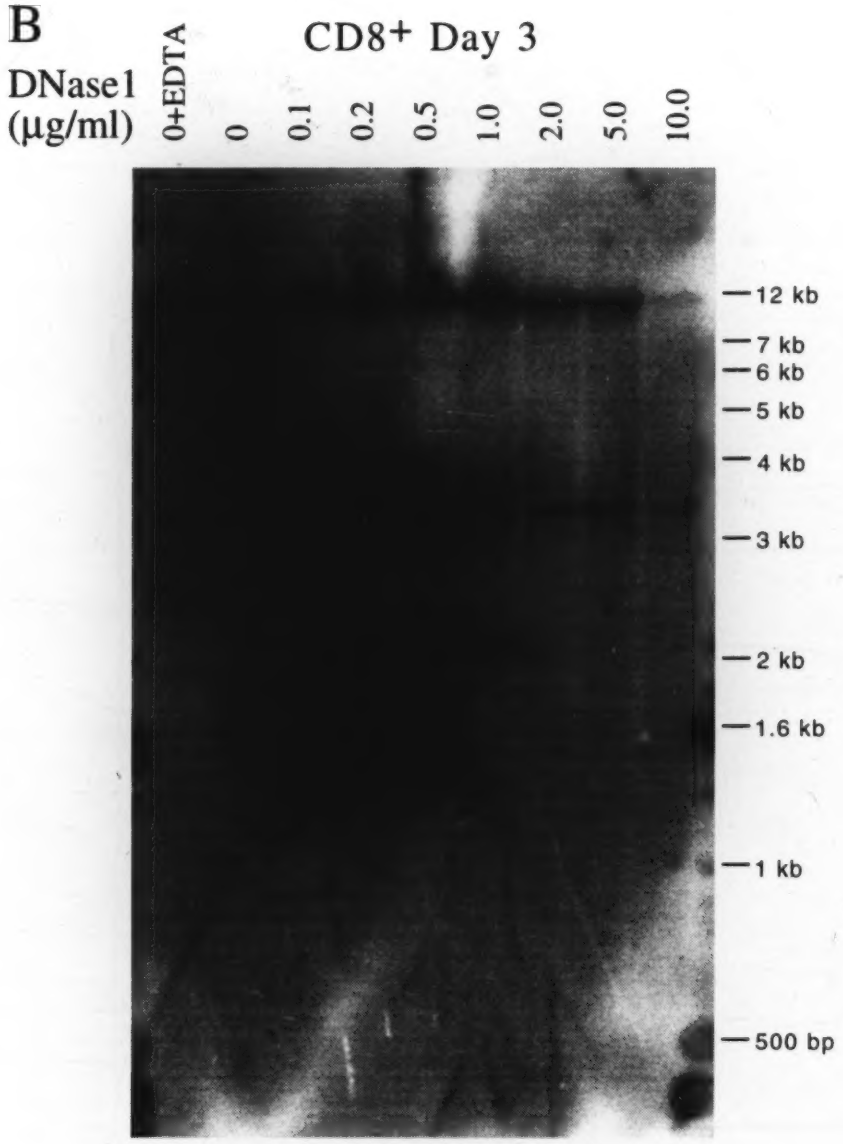

A
CD8+ Day 3
DNase1 (µg/ml)
0+EDTA
0
0.1
0.2
0.5
1.0
2.0
5.0
10.0
6 kb
5 kb
4 kb
Hypersensitive Site
3 kb
2 kb
1.6 kb
1 kb
500 bp

Figure 3-9


B
CD8+ Day 3
DNase1 (µg/ml)
0+EDTA
0
0.1
0.2
0.5
1.0
2.0
5.0
10.0
12 kb
7 kb
6 kb
5 kb
4 kb
3 kb
2 kb
1.6 kb
1 kb
500 bp

methylation (Fig. 3-10A). One such footprint corresponds to the AP1 binding site and two others corresponding to the CBF binding sites. The footprint at the CRE is indicated by a reduction of the band correlating to the G residue midpoint in the binding site. Two hypersensitive bands were observed corresponding to the A residues that flank the Ikaros sequence element (Georgopoulos *et al.*, 1992).

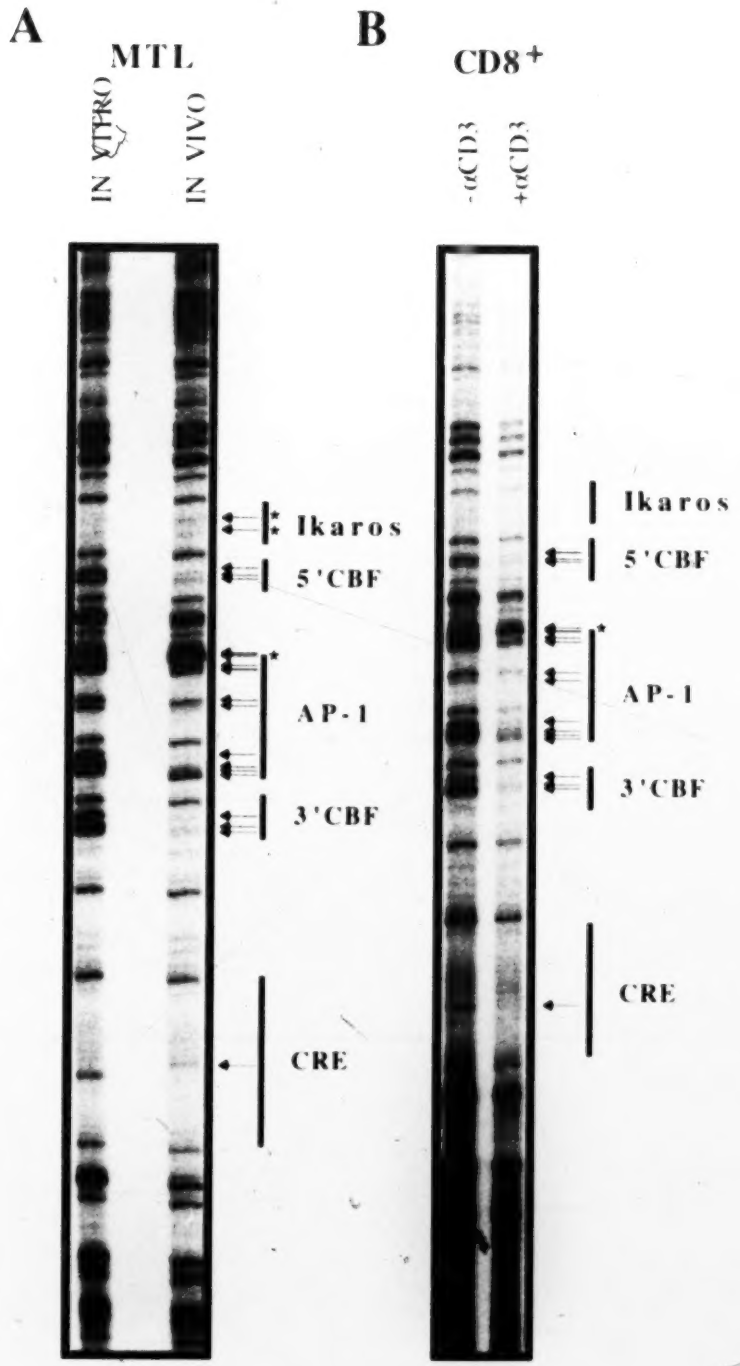
$CD8^+$ splenocytes isolated by passage over a CD8 immunocolumn were treated directly with DMS or stimulated for 48 hours with αCD3 and then subject to DMS treatment. A comparison of the resting and activated lanes in Figure 3-10B shows that only in activated $CD8^+$ cells did footprints appear at the AP1, CRE, and both CBF sites. A hypersensitive site was detected at the 5' boundary of the AP1 recognition sequence. Bands corresponding to the internal G residues of both the AP1 and CBF sites were reduced. The hypersensitive A residues of the Ikaros element observed in MTL 2.8.2. were not apparent, probably due to the resolution limits of the gel. The in vivo footprints correlate well with the in vitro data and present a snapshot of the endogenous gene as it is being transcribed. The absence of proteins bound in resting cells suggests that the transcription factors that are present do not have access to the DNA and moreover it suggests a lack of repressor interactions. The resting cell profile was invariably identical to the naked DNA control in all experiments performed. In non-expressing L cells, no protein-DNA interactions were observed (data not shown). This data indicates that the AP1, CBF, and CRE binding sites are not occupied by their respective transcription factors in resting splenocytes and that T cell activation is required for DNA binding.

Figure 3-10 In vivo footprinting analysis of the granzyme B promoter shows proteins bound at the CRE, AP1, Ikaros, and core binding factor sites in MTL 2.8.2 and in activated CD8+ T cells.

Genomic DNA was isolated following DMS treatment of cells and subject to piperidine cleavage and ligation-mediated PCR as described in materials and methods. The PCR products were separated on a 7%, 7M Urea polyacrylamide gel. A footprint is observed as one or more bands that are reduced or increased in intensity relative to the control DNA. This work was performed by Brenda L. Duggan.

A. The DMS cleavage-LMPCR pattern of naked DNA (in vitro) and MTL DNA (in vivo). The bands that show evidence of protein binding are indicated with arrows in the in vivo lane. Bands that are reduced in intensity appear at the 5' and 3' CBF elements, at the AP1, and CRE elements. Hypersensitive bands are denoted with an asterisk in the AP1 and Ikaros binding sites.

B. The -αCD3 lane shows the DMS cleavage-LMPCR pattern of unstimulated $CD8^+$ DNA and it is identical to the in vitro lane above in A. The +αCD3 lane shows the cleavage pattern obtained for stimulated (two days in the presence of αCD3 and IL-2) $CD8^+$ DNA and protected residues are indicated with arrows on the right. A hypersensitive band is indicated at the 5' boundary of the AP1 element.


A
MTL
IN VITRO
IN VIVO
Ikaros
5'CBF
AP-1
3'CBF
CRE
B
CD8+
-αCD3
+αCD3
Ikaros
5'CBF
AP-1
3'CBF
CRE

DISCUSSION

Each peripheral cytotoxic lymphocyte spends its life in a continuous search for the foreign antigen that can turn it into a potent killer. The acquisition of cytotoxic function requires de novo synthesis and assembly of the killing machinery. The granzyme B gene encodes one of the components of this machinery and is fully activated within three days. The major thrust of our experiments has been directed at understanding granzyme B gene induction in physiologically relevant primary lymphocytes.

We have used purified $CD8^+$ splenocytes in reporter gene transfections, DNase1 hypersensitivity analysis, mobility shift assays, and in vivo footprinting. There was a recapitulation of general motifs that have been noted in regulation studies of other inducible or developmentally regulated genes. *Cis*-acting transcriptional enhancers of tissue-specific genes tend to consist of a cluster of ubiquitous and tissue-specific transcription factor binding sites. The granzyme B promoter region contains binding sites for the widely expressed AP1 and CRE transcription factors as well as for the T cell-specific core binding factor and Ikaros. These sequences can bind to nuclear proteins present in activated T cells and are sufficient to activate high levels of reporter gene expression in transient transfections. These sequences are important for granzyme B regulation in vivo as every binding site is fully accessible to, and bound by, transcription factors in activated $CD8^+$ T cells. The spacing of these sequences is interesting in that the close proximity may be necessary for the concerted enhancer affect of several differentially regulated factors at this specific locus.

These trans-acting factors must be activated at the appropriate time by developmentally or externally derived signals. The Ikaros proteins are expressed throughout T cell development. We have shown that they are present in resting $CD8^+$ splenocytes and capable of binding DNA. Our data also shows Ikaros to be bound in vivo in MTL 2.8.2 cells. We showed by EMSA and in vivo footprinting that the AP1 transcription factor does not bind to its sequence element in the granzyme B promoter

without prior activation through the T cell receptor. The transcription factor AP1 is comprised of Fos and Jun heterodimers whose activity is regulated by de novo synthesis as well as post translational modification. Upon activation through the T cell receptor, they are phosphorylated through a protein kinase C/Ca^{+}-mediated signal transduction cascade (reviewed by Crabtree and Clipstone, 1994; Karin and Smeal, 1992; Rincon and Flavell, 1994). The phosphorylated AP1 complex can efficiently bind to its cognate DNA sequence element and act as a potent transactivator of transcription. Core binding factor has been implicated in the regulation of many T cell-specific genes such as the TCR α, β and δ genes, the CD3 ε and δ genes (Hallberg *et al.*, 1992; Hsiang, 1993; Prosser, 1992). CBF consists of two heterologous subunits, a DNA binding α subunit and a non-DNA binding β subunit (Wang *et al.*, 1993; Zaiman *et al.*, 1995). Through heterodimerization, the β subunit augments the DNA binding affinity of the α subunit. The α subunit has been shown to localize to the nucleus while the β subunit is found in the cytoplasm (Lu *et al.*, 1995). Little is known about the signaling events that lead to the translocation of the α/β heterodimer into the nucleus. The hypothesis put forth suggests that the α subunit requires modification to make it more amenable to association with the β subunit. This modification would occur after the cell has been activated through the appropriate cell surface receptors. Results of mobility shifts show a weak complex in resting cells that could indicate a low affinity, partially dissociated α subunit complex. The complex in activated cells was much more intense and well defined.

Both CBF and AP1 are able to act in combination with other transcription factors, most notably ets and NF-AT (nuclear factor of activated T cells) (Jain *et al.*, 1993; Wotton *et al.*, 1994). In the regulation of the IL-2 gene, NF-AT translocates to the nucleus in response to increases in intracellular levels of calcium. The nuclear NF-AT then unites with activated Fos/Jun to form a complex with high affinity DNA binding and transactivation properties. Recently it has been determined that there is more than one form of NF-AT (Northrop *et al.*, 1994). Core binding factor is encoded by members of a

multigene family, and one member of the α subunit family has been shown to be T cell specific (Satake *et al.*, 1995). An attractive model, similar to IL-2 gene induction, is that upon T cell activation CBF and AP1 are modified by separate signaling pathways and then unite at the level of DNA binding and transactivation.

Chromatin is no longer viewed as a passive participant in eukaryotic gene regulation. Activated *trans*-acting factors must be allowed access to their target *cis*-enhancer elements only in the appropriate tissues and at the appropriate times. The formation of nuclease hypersensitive sites in chromatin has been correlated with such important regulatory elements as enhancers, silencers, and locus control regions (reviewed by Felsenfeld, 1993; Gross and Garrard, 1988). For example, the human β-globin locus is composed of five developmentally regulated genes that are induced and expressed sequentially during embryonic, fetal, and adult development. The timely expression of these genes is controlled by a series of four stage-specific DNase1 hypersensitive sites that exist many kilobases upstream of the 5' most gene in the cluster (Frazer *et al.*, 1993). These hypersensitive sites consist of binding sites for ubiquitous and erythroid-specific transcription factors. Their stage-specific appearance has been shown to be dependent upon interaction with individual gene promoters of the globin genes (Reitman *et al.*, 1993). It appears in this case that a cluster of binding sites alone is not sufficient to create a hypersensitive site but an interaction with a distant element is necessary to induce structural changes in the locus.

The granzyme gene locus is potentially very interesting in this respect. We do not know whether the granzyme B proximal promoter sequences alone are sufficient to create the observed hypersensitive site in activated T cells or if another sequence element located elsewhere is required. The possibility may exist that all or a subset of the granzyme genes could be coordinately regulated by a higher level of control that involves the interaction of the individual promoters with a distant locus control region that would make the genes amenable to transcription upon the reception of the appropriate induction signal. We have

looked for additional hypersensitive sites up to 5 kb upstream, 9 kb downstream and within the gene and found none.

There is increasing evidence that in vitro footprinting assays do not always reflect the true DNA/protein interactions occurring in the chromatin of intact cells. The state of chromatin condensation may sequester *cis*-elements and prevent binding of available transcription factors. In addition, the non-expressing cells showed no evidence of a DNase1 hypersensitive site in the vicinity of the granzyme B gene. Interestingly, a hypersensitive site was apparent in activated but not resting $CD8^+$ lymphocytes. An inducible hypersensitive site has been observed at the human granzyme B promoter in a PEER T cell line when activated with TPA and dibutyryl-cAMP (Hanson *et al.*, 1990). This site extends from approximately 30 to 400 nucleotides upstream of the transcription start site and roughly corresponds to the promoter sequences that are highly conserved between mice and humans. We were able to define more precisely the limits of the murine hypersensitive site in activated splenocytes and show that transcription factors form sequence-specific footprints at this site in vivo.

This series of experiments, together with the Northern analysis, transfection, and EMSA data, leads us to infer that the granzyme B locus may not be accessible to transcription factors in resting T cells or non-T cells. Ikaros and CRE binding proteins are present in resting cells but only upon activation of the T cells are the AP1 and CBF capable of binding to their cognate sequence elements and, at this time, the promoter becomes accessible to them. There is presumably a highly coordinated mobilization of factors once the decondensation signal takes place. The promoter must be sensitive to multiple signaling events and it is possible that the assembly of factors only occurs after all of the signaling events have been achieved. The IL-2 enhancer displays an all or nothing chromosomal binding phenomenon in EL4 cells stimulated with TPA/A23187 (Garrity *et al.*, 1994). When cyclosporin A treatment was used to block the activation of the calcium-dependent NF-AT and NF-κβ, transcription factors that normally bind to the IL-2 enhancer, no

binding of even cyclosporin insensitive factors was observed in vivo and the enhancer was unoccupied.

It has yet to be determined whether the transcription factor binding sites become accessible because of a shift in the nucleosome structure at the promoter or if the transcription factors themselves mediate the formation of the hypersensitive site. The granzyme B locus is relatively insensitive to DNase1 digestion in L cell nuclei. This is not surprising because at no time in a fibroblast's existence does it produce granzyme B. Histone modifications, such as hyperacetylation of histone tails, or nucleosomal binding proteins such as nucleoplasmin and SWI/SNF (Chen *et al.*, 1994; Kwon *et al.*, 1994; Wolffe, 1994; Workman and Buchman, 1993) are potentially important players in gene induction. These proteins have been shown to facilitate transcription factor binding and nucleosome disruption and may be essential components of the chromatin rearrangement and transcriptional induction processes. Whether these molecules or others play a role in granzyme B regulation in vivo awaits further investigation.

In this investigation we have confirmed that more than one level of regulation is required to permit the expression of granzyme B. Our proposed model of granzyme B regulation involves decondensation at the chromosomal locus in response to T cell activation. The integration of multiple signals would culminate in the synthesis or activation of the necessary transcription factors and result in a staged assembly of factors at the newly accessible granzyme B promoter. The subsequent three dimensional structure would then activate the basal transcription machinery to initiate transcription and elongate the nascent RNA. This is similar to the model proposed for the regulation of IL-2 transcription (Garrity *et al.*, 1994). Both systems require timely activation in response to lymphocyte stimulation and must be readily reversible in order to terminate the immune response.

CHAPTER 4[1]

MUTATIONAL ANALYSIS OF THE MURINE GRANZYME B GENE PROMOTER IN PRIMARY LYMPHOCYTES AND A T CELL CLONE

INTRODUCTION

Several distinct signal transduction pathways are activated via the T cell receptor and costimulatory molecules. These signals are integrated in the nucleus in the form of a particular subset of activated transcription factors that converge on a specifically targeted promoter and activate transcription. By studying the specialized requirements for T cell-specific gene expression in terms of transcription factor necessity we can further understand the molecular events that are associated with T cell activation.

Site directed mutagenesis is a powerful biological tool that precisely alters DNA sequences in order to study a particular region of interest in protein or nucleic acid function. This method was utilized to determine whether the transcription factor binding sites discussed in the previous chapter were required for granzyme B transcription. Three point mutations were systematically introduced into the CRE, AP1, Ikaros and the two CBF binding sites, in the context of the 243 bp promoter. In addition, several double mutant promoters and a quadruple mutant promoter, in which only the CRE was intact, were constructed and the series was assessed for their effect on transcription in T cells and non-T cells.

We analyzed our promoter mutations by transient transfection assays in two distinct populations of T cells, freshly activated primary lymphocytes and an IL-2-dependent T cell clone (MTL). Primary lymphocytes were activated by a variety of stimulation conditions and then transfected. MTL cells constitutively express the granzymes and transfections

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[1] A version of this chapter has been published: Babichuk, C.K. and R.C. Bleackley. 1997. *J. Biol. Chem.* 272:18939.

were performed without any additional stimulation other than IL-2. Whether this immortalized state of activation reflects the state of primary activation is unknown; however, our transfection studies in these distinct cell types revealed some differences. Finally, we investigated how the mutant and wild type promoters functioned in L cell fibroblasts. These experiments enabled us to observe the effects of removing each transcription factor from the native promoter complex and assess its importance in the regulation and specificity of the granzyme B gene.

RESULTS

The base pair changes that were introduced were primarily transversions in the consensus sequence binding sites and were chosen on the basis of previous methylation interference data, in vivo footprinting data, and site directed mutagenesis data collected either on the granzyme B promoter or on similar binding sites in other gene promoters (Figure 4-1). This strategy conserved the spacing and the helical orientation of the binding sites. Following the mutagenesis reactions each construct was confirmed by sequencing and subcloned into a promoterless luciferase reporter gene plasmid. With the exception of the CRE and 5' CBF, each mutant binding site was tested by electrophoretic mobility shift competition assays for the inability to compete for the wild type binding site with up to a 200 molar excess of mutant probe.

Mutational Analysis of the Murine Granzyme B Promoter in Stimulated Primary Lymphocytes.

We have shown that activated $CD8^+$ cells isolated by immunomagnetic separation were able to drive high levels of reporter gene expression from the granzyme B promoter in transient transfections. Typically, the T cell portion of a whole splenocyte population expanded in culture to approximately 75% following stimulation with αCD3 or con A for three days, as assessed by FACS analysis (unpublished observations). The $CD8^+$

Figure 4-1

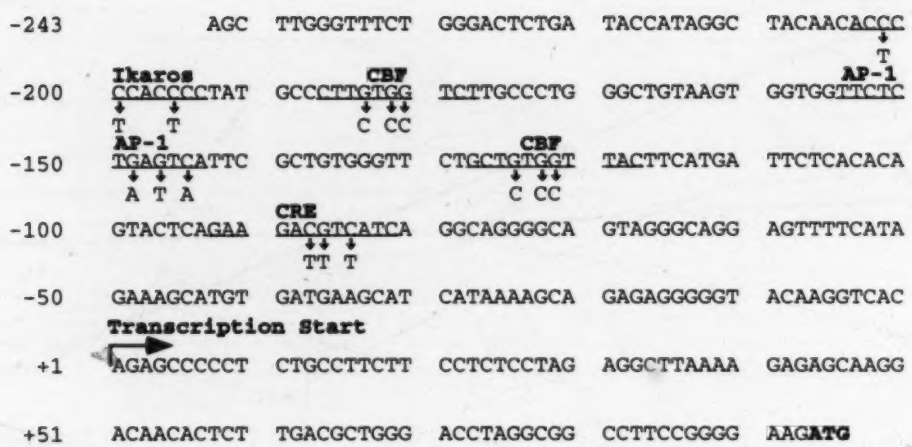

-243 AGC TTGGGTTTCT GGGACTCTGA TACCATAGGC TACAACACCC
Ikaros CBF AP-1
-200 CCACCCCTAT GCCCTTGTGG TCTTGCCCTG GGCTGTAAGT GGTGGTTCTC
T T C CC T
AP-1 CBF
-150 TGAGTCATTC GCTGTGGGTT CTGCTGTGGT TACTTCATGA TTCTCACACA
A T A C CC
CRE
-100 GTACTCAGAA GACGTCATCA GGCAGGGGCA GTAGGGCAGG AGTTTTCATA
TT T
-50 GAAAGCATGT GATGAAGCAT CATAAAAGCA GAGAGGGGGT ACAAGGTCAC
Transcription Start
+1 AGAGCCCCCT CTGCCTTCTT CCTCTCCTAG AGGCTTAAAA GAGAGCAAGG
+51 ACAACACTCT TGACGCTGGG ACCTAGGCGG CCTTCCGGGG AAGATG

Figure 4-1 The nucleotide sequence of the proximal granzyme B promoter and the nucleotide substitutions present in the transcription factor binding sites.

Major potential transcription factor binding sites are underlined and the transcription factor designations are shown above the sequences (CRE,cyclic AMP responsive element; CBF, core binding factor; AP-1, activator protein-1). Specific nucleotide substitutions are indicated by the arrows below the sequence for each binding site. The transcription start site is indicated by an arrow and the translation initiation start site(ATG) is indicated in boldface.

population proliferated and expanded to approximately 50% of the total culture, whereas the $CD4^+$ population remained constant at approximately 25%. Most of the non-T lymphocyte population did not survive in this type of culture and we do not believe that they contributed significantly to reporter gene expression observed from the granzyme B promoter.

The promoter/luciferase plasmid series was transfected, along with a β-galactosidase control plasmid, into whole primary lymphocyte cultures that were stimulated for one day prior to transfection and for two days following. The cells were harvested and assayed for luciferase and β-galactosidase activities. The luciferase reporter gene activity was significantly high in primary cells with the average Relative Light Unit (RLU) value for the full length 828 bp promoter being over 2000 and background (promoterless plasmid) being approximately 200 RLU. Also, β-galactosidase activities were very reproducible within each transfection experiment.

The averaged corrected values for two independent primary cell transfection experiments are shown in Figure 4-2. These cells were stimulated with αCD3, con A, and IL-2. The values on the y-axis represent the RLU of the luciferase assay divided by the absorbance of the β-galactosidase assays x 1000. Because the β-galactosidase expression and incubation times of the assay differ between each transfection, the corrected values are only relative within a single experiment and are not compared between experiments or cell types. The promoterless pGL2 basic vector served as the negative control and gave luciferase values that were essentially background. The next three bars depict the promoter deletion series -108, -169, and -243. As transcription factor binding sites were added by increasing the length of the promoter to 243 bp, the luciferase activity increased substantially. The next eight bars represent the mutant promoter series in the context of the 243 bp promoter. The largest effect for any single point mutation was observed for the CRE binding site, which lowered total promoter activity to 60 % of wild type. A surprising finding was that the mutation of the AP1 binding site appeared to have relatively

Figure 4-2

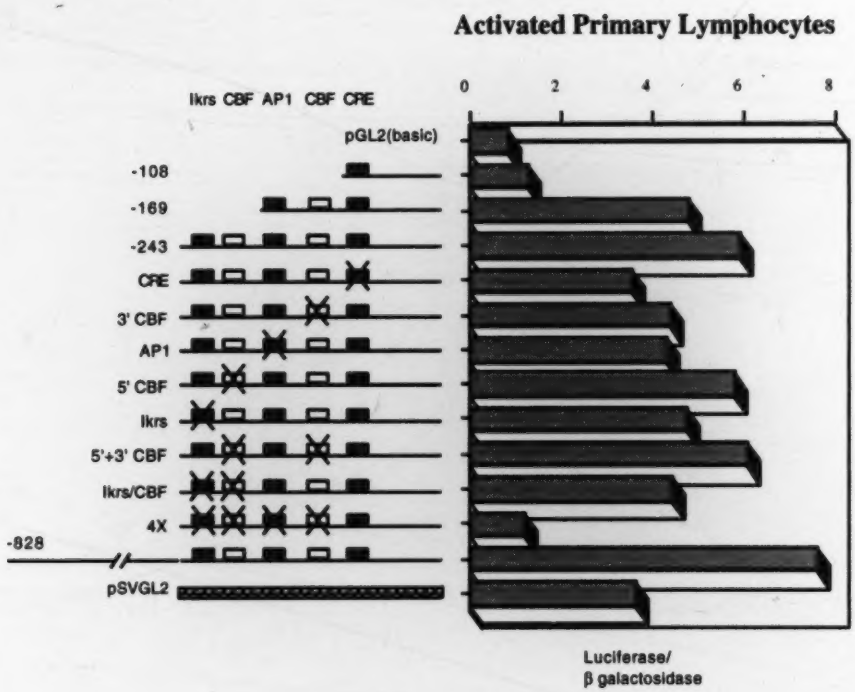


Figure 4-2 Transient transfection analysis of granzyme B promoter mutations in primary lymphocytes stimulated with αCD3, con A, and IL-2.

Three deletion fragments and a series of transcription factor binding site mutants were cloned into the pGL2 promoterless luciferase reporter gene plasmid and were transfected along with an SVβ-galactosidase control plasmid into primary lymphocytes. Cells were harvested after two days and assayed for reporter gene activity. The values represent an average of two independent transfections and are depicted as the relative light unit value of the luciferase assays divided by the A420 value of the β-galactosidase assays. These values are: pGL2 0.45, 0.39; -108 0.79, 0.51; -169 3.2, 1.59; -243 2.7, 3.18; CRE 1.82, 1.76; 3'CBF 1.73, 2.68; AP1 2.17, 2.16; 5'CBF 3.09, 2.72; Ikrs 1.99, 2.77; 5'+3'CBF 2.94, 3.13; Ikrs/5'CBF 1.97, 2.49; 4X 0.62, 0.59; -828 3.58, 4.01; SVGL2 1.97, 1.67. The maximum averaged standard deviation of the luciferase assays(performed in triplicate) for the two experiments was ± 0.335 (5' CBF/Ikaros) and the minimum was ± 0.046 (pGL2). Promoter fragments and transcription factor binding sites are diagramatically represented on the left with boxes and binding site mutations are indicated with an X.

little impact on overall promoter activity (73% of wild type). Mutations in the 3' CBF or Ikaros binding sites had slight negative effects on transcription whereas the mutation of the 5' CBF site had no effect on promoter activity. Overall, none of the single binding site mutations reduced activity to an extent where it was possible to conclude that any one factor was necessary for the expression of the 243 bp granzyme B promoter.

We then looked at the mutant binding sites in combination. Promoters lacking either, or both of the 5' and 3' CBF binding sites consistently showed normal expression in transient assays. The 5' CBF/Ikaros double mutant contained the same binding site complement as the -169 promoter and was approximately as active. When the AP1, Ikaros, and both CBF elements were all ablated the result was a very dramatic decrease in overall activity, comparable to the expression observed from the -108 promoter. Therefore, no other elements distal to the CRE, other than the ones that were identified, were involved in promoter transcription.

Primary Lymphocytes Treated with Individual Stimuli Displayed Similar Expression Patterns of the Mutant Promoters

Our primary cell cultures were initially stimulated with a combination of three potent activators of T cells (αCD3, con A, and IL-2) in an effort to maximize granzyme B-luciferase reporter gene expression. This treatment, however, may mask contributions to overall promoter output by the individual transcription factors. The αCD3 antibody utilized in these experiments binds to the ε chain of the CD3/TCR complex (Leo *et al.*, 1987). This antibody, in combination with IL-2, is a sufficient stimulus to generate potent cytolytic activity in primary lymphocyte cultures. Con A is a widely employed mitogen that interacts non-specifically with surface glycoproteins and is capable of activating T cells through the T cell receptor and likely through other glycosylated surface receptors. At high doses, IL-2 activates primary T lymphocytes to express perforin and granzyme B mRNA's and these cells possess cytotoxic potential (Liu *et al.*, 1989; Smyth *et al.*, 1990). Instead of using all

three stimuli simultaneously, αCD3, con A, and IL-2 were added in culture individually, or in combination, and the mutant promoter fragments were transfected as above and assessed for reporter gene expression.

Con A alone or with added IL-2 appeared to be the best stimulus for this type of culture as the cells proliferated well and expressed high levels of luciferase activity from the granzyme B promoters. Figure 4-3 depicts the average expression profiles of the wild type and mutant granzyme B promoters in con A and con A + IL-2 stimulated whole primary lymphocytes. In cells treated with con A alone (panel A), mutations in the CRE and AP1 binding sites lowered overall promoter expression to approximately 60 % of wild type. The Ikaros mutation and either CBF binding site mutations were somewhat inhibitory to promoter activity. Only the 4X mutant promoter, with mutations in the AP1, Ikaros, and both CBF sites significantly decreased promoter activity to near background levels, similar to the 108 bp promoter fragment (data not shown). No major effect of any individual binding site mutations was evident in these transfection experiments with con A stimulation alone.

Continuous exposure to 60 U/ml of exogenous IL-2 in addition to con A did appear to enhance the effects of certain mutations slightly. Under these conditions, mutations in the CRE and AP1 binding sites lowered overall promoter activity to 54% and 40% of wild type, respectively, the greatest extent observed in any of the primary cell transfections (Fig. 4-3B). The Ikaros and 5' CBF were dispensable to promoter function in these transfections but the 3' CBF site may have been somewhat important as the expression of this mutant was only slightly higher than that of the CRE mutant.

Transfection experiments with cells stimulated with αCD3 alone resulted in a very good transfection efficiency, as determined by the high levels of β-galactosidase expression from the control plasmid, but very little granzyme B promoter activity was observed from any of the mutant or wild type promoters (data not shown). The combination of αCD3 and IL-2 did stimulate the cells enough to detect significant luciferase levels when

Figure 4-3

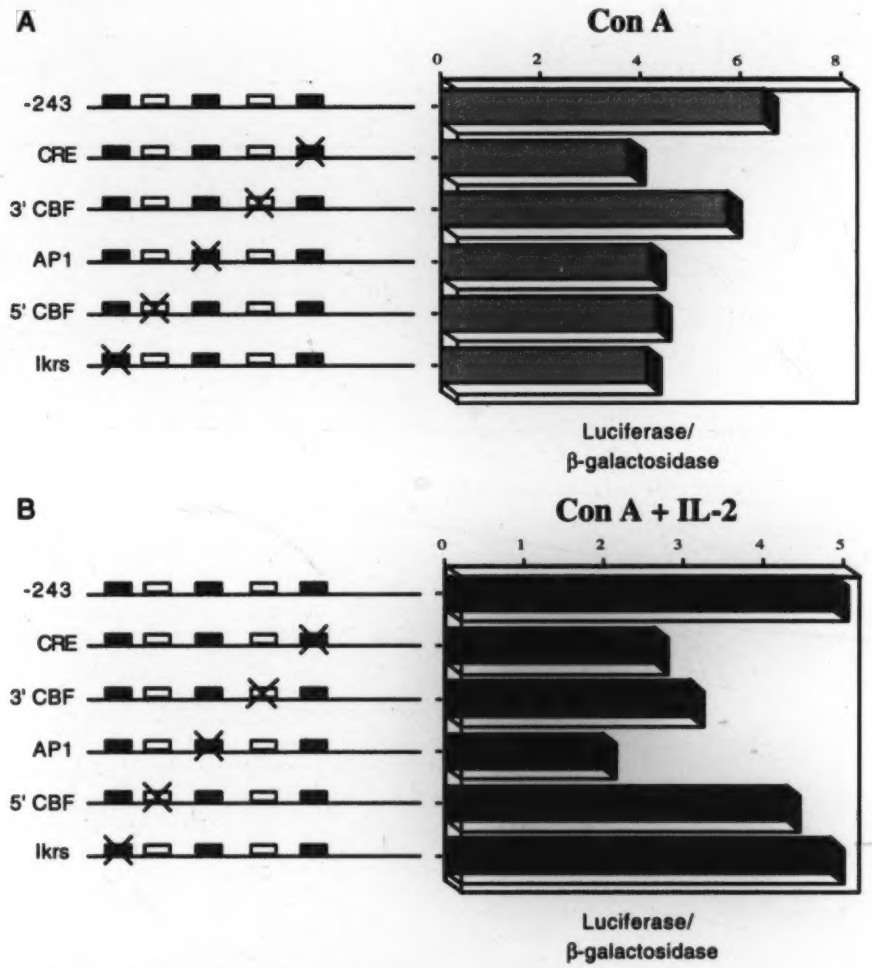


Figure 4-3 Effects of individual binding site mutations in primary lymphocytes stimulated with con A alone or con A + IL-2.

Primary lymphocytes were stimulated for one day and transfected with the wild type -243 granzyme B promoter and each single binding site mutant promoter. Values represent the average of two independent transfections. A, lymphocytes cultured in 5 μg/ml con A. These values are: -243 2.78, 3.639; CRE 1.18, 2.575; 3'CBF 1.82, 3.88; AP1 1.75, 2.431; 5'CBF 2.19, 2.122; Ikaros 1.19, 2.891. B, lymphocytes cultured in 5 μg/ml con A and 60 U/ml IL-2. These numbers are: -243 2.738, 2.11; CRE 1.271, 1.33; 3'CBF1.649, 1.42; AP1 0.923, 1.04; 5'CBF 2.356, 1.9; Ikaros 2.044, 2.76. The maximum averaged standard deviations of the luciferase assays (performed in triplicate) for the two experiments were ± 0.437 (-243) for con A stimulated cells and ± 0.203 (-243) for con A + IL-2 stimulated cells.

approximately twice the normal number of cells were transfected. The expression pattern was very similar to that obtained with con A stimulation. The CRE and AP1 sites were most important but neither mutation lowered promoter activity to a significant extent and the Ikaros and both CBF binding sites were dispensable (data not shown).

Finally, primary lymphocytes were treated with 5000 U/ml of human recombinant IL-2 and transfected with the longer 828 bp promoter after 20 hours of initial stimulation. After further IL-2 treatment, no luciferase activity and only trace amounts of β-galactosidase activity were detectable following one, two, or three days post transfection and the cells failed to proliferate at any significant rate. It is likely that these cells were resistant to transfection and were unable to express the reporter gene.

Analysis of the Mutant Promoters in MTL 2.8.2 Cells

MTL 2.8.2 is an immortalized, IL-2-dependent, cytotoxic T cell clone that constitutively expresses the endogenous granzymes. These cells were transfected with the wild type and mutant promoter constructs and assayed for expression (Figures 4-4). The absolute luciferase values were higher in MTL than in primary cells with the RLU reading for the full length 828 bp promoter greater than 10,000. The pGL2 promoterless vector served as the negative control and was essentially background. The activities of the minimal promoter deletion series are depicted in the next three bars. Luciferase activity increased proportionately as the length of the promoter was extended to 243 bp. Of the five individual promoter mutants (next five bars), the CRE binding site appeared to be the most important controlling factor (36% of wild type activity). The AP1 site was relatively important as well in that the promoter was transcribed at 64% of wild type levels in the absence of AP1 binding. Mutation of the 3' CBF site had no effect on the promoter and the 5' CBF site had, if anything, a stimulatory effect in both the single mutant and the double CBF mutant. Neither the 5' CBF or Ikaros mutation alone was inhibitory, but together they reduced expression to below that of the -169 promoter, to near background levels.

Figure 4-4

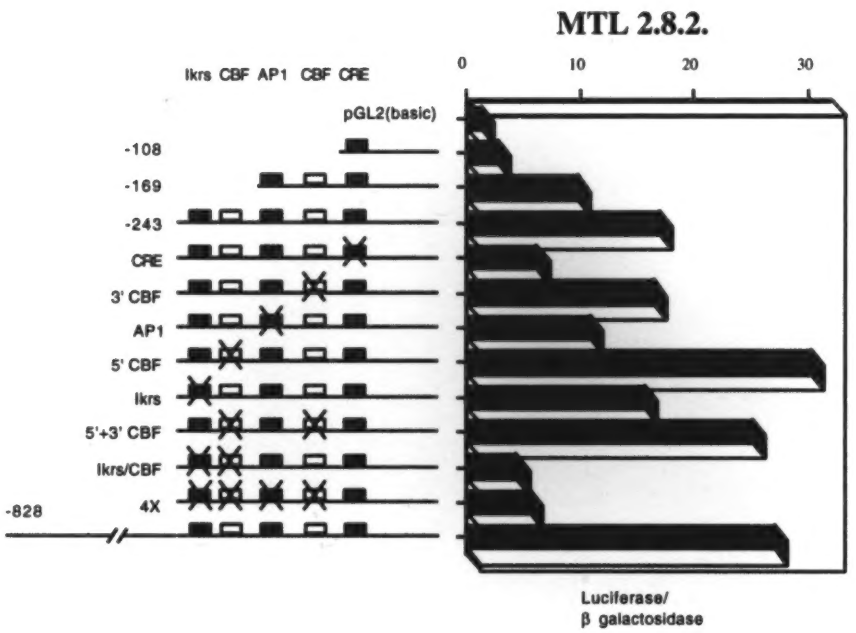


Figure 4-4 in Transfection analysis of granzyme B promoter mutations an IL-2 dependent murine CTL line, MTL 2.8.2.

The same constructs as shown in Fig. 4-2 were transfected into MTL 2.8.2 cells cultured in IL-2 containing media (60 U/ml) and harvested after two days. The values represent the average of two independent transfections and are depicted as luciferase/β-galactosidase. These values are: pGL2 0.47, 0.69; -108 1.54, 1.03; -169 6.03, 3.61; -243 9.36, 7.47; CRE 3.53, 2.55; 3'CBF 10.61, 5.82; AP1 5.57, 5.26; 5'CBF 20.25, 9.94; Ikaros 10.54, 5.07; 5'+3'CBF 16.98, 8.09; 5' CBF/Ikrs 2.3, 2.02; 4X 3.92, 1.63; -828 16.52, 10.4; SVGL2 43.68, 16.95. The maximum averaged standard deviation of the luciferase assays (performed in triplicate) for the two experiments was ± 1.69 (5' CBF) and the minimum was ± 0.061 (pGL2). Transcription factor binding sites are represented by boxes on the left and mutations are indicated with an X.

This result was not observed in primary lymphocytes. As expected, the 4X mutant promoter was expressed at near background levels and was almost as low as its functional equivalent, the 108 bp promoter. In MTL cells, the CRE was the most important individual controlling element and both the deletion analysis and the site-specific mutagenesis data indicate an important role for Ikaros and CBF in granzyme B expression.

AP1 In Conjunction with 3' CBF is Important in Conferring High Levels of Granzyme B Promoter Activity in T Cells

It was observed that when the AP1, Ikaros, and both CBF binding sites were abolished, expression dropped to near background levels. However, neither of the CBF sites appeared to be important for the observed high activity of the 243 bp fragment. To determine whether the AP1 and Ikaros binding sites were responsible for this dramatic loss of activity, a double mutant promoter was constructed in which both the AP1 and Ikaros sites were abolished. Additionally, when the AP1 and 3' CBF sites were added to the -108 promoter (the -169 fragment), activity increased substantially, indicating that these two factors may be very important for promoter activity. A second double mutant promoter was constructed in which both the AP1 and 3' CBF sites were abolished.

The reporter gene activity of these new mutant constructs was compared to the wild type promoter, each single mutant, and the 4X mutant promoter. The averaged values for four independent transfection experiments in activated primary cells and two independent transfections in MTL cells are shown in Figure 4-5. In primary cells, the AP1 mutation appeared to reduce promoter activity to 61% of wild type and Ikaros had no inhibitory effect (panel A). The AP1 mutant was much less active in MTL cells (37% of wild type) and some inhibition was observed for the Ikaros mutant (panel B). When both the AP1 and Ikaros binding sites are abolished, promoter expression was reduced to an extent in primary cells (47% of wild type) and was approximately the same as the AP1 mutant in MTL (40%). When the AP1 and 3' CBF binding sites are abolished, the promoter is still

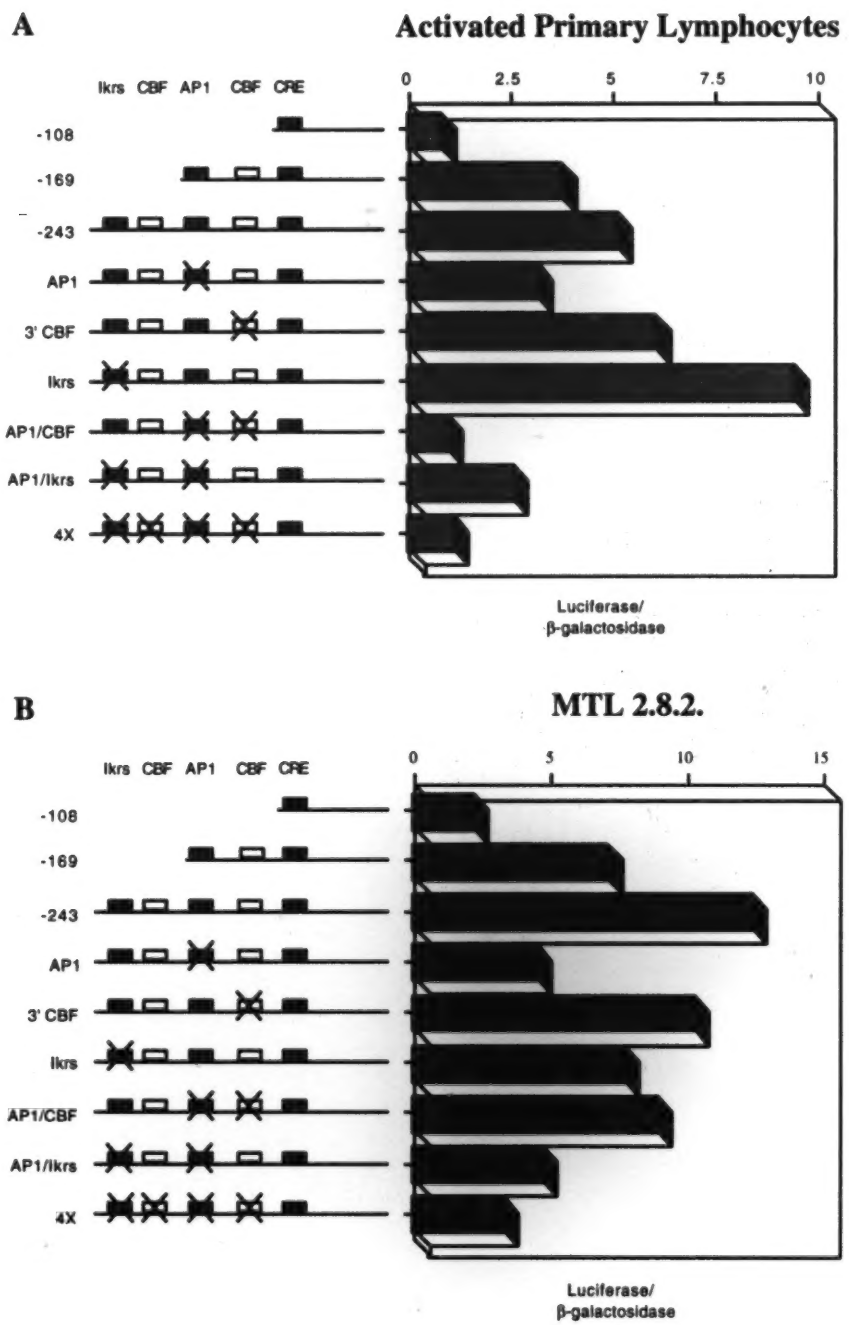
Figure 4-5 The AP1 and the 3' CBF binding sites together are important for the high levels of expression of the -243 promoter.

A. Primary lymphocytes were activated with αCD3, con A, and IL-2 and were transfected with the wild type, each individual, the new double mutant promoters and the 4X mutant promoter.

B. The same constructs and above were transfected into MTL 2.8.2. cells.

Both graphs represent the average values obtained from at least two independent transfection experiments and are depicted as luciferase/β-galactosidase. The values obtained in primary cells for the individual transfections were: -108 0.19, 0.191, 0.108, 0.11; -169 0.708, 1.455, 0.389, 0.404; -243 1.573, 1.066, 0.852, 0.574; AP-1 0.875, 0.792, 0.569, 0.273; 3'CBF 2.673, 1.124, 0.745, 0.251; Ikaros 2.759, 3.59, 0.829, 0.307; AP-1/3'CBF 0.245, 0.169, 0.191, 0.136; AP-1/Ikrs 0.931, 0.439, 0.383, 0.251; 4X 0.372, 0.245, 0.16, 0.092. The values obtained in MTL cells were: -108 1.053, 1.092; -169 3.594, 3.452; -243 7.354, 4.983; AP1 2.507, 1.964; 3'CBF 5.636, 4.592; Ikrs 3.892, 3.759; AP-1/3'CBF 5.129, 3.76; AP-1/Ikrs 2.349, 2.325; 4X 1.499, 1.764. The averaged maximum standard deviations of the luciferase assays (performed in triplicate) for each experiment were ± 0.176 (3' CBF) for the primary cells and ± 0.342 (-243) for the MTL cells.

Figure 4-5


A
Activated Primary Lymphocytes
Ikrs CBF AP1 CBF CRE
-108
-169
-243
AP1
3' CBF
Ikrs
AP1/CBF
AP1/Ikrs
4X
0
2.5
5
7.5
10
Luciferase/
β-galactosidase
B
MTL 2.8.2.
Ikrs CBF AP1 CBF CRE
-108
-169
-243
AP1
3' CBF
Ikrs
AP1/CBF
AP1/Ikrs
4X
0
5
10
15
Luciferase/
β-galactosidase

active in MTL cells (73%), despite the absence of a functional AP1 binding site. However, expression is reduced to background levels (20%) in primary cells indicating that together these sites are very important in conferring high transcriptional activity. From this series of transfections we conclude that the presence of functional binding sites for AP1 and the 3' CBF is primarily responsible for the high levels of expression observed from the granzyme B minimal promoter in primary lymphocytes. MTL cells, however, were primarily sensitive to mutations in the AP1 binding site alone but not in combination with the 3' CBF.

Expression of the Reporter Gene in L Cells is Insensitive to Any of the Binding Site Mutations

L cells are a murine fibroblast line which have been found to express several of the transcription factors believed to be important for granzyme B expression. These include CRE, AP1, and a ubiquitously expressed member of the core binding factor family. Ikaros is not present in fibroblasts as its expression is restricted to lymphoid cells. L cells contained no detectable levels of granzyme B mRNA yet they still produced relatively high reporter gene expression from the granzyme B minimal promoter in transient transfection assays. To determine whether any of these binding sites were responsible for, or were important to, the granzyme B promoters' activity in non-T cells, the series of mutant and wild type promoters were transfected into L cells. None of the single binding site mutations caused any dramatic reduction in expression compared to the wild type promoter (Figure 4-6). This observation implied that no one factor was responsible for activating transcription from this promoter in L cells. Of the three double mutants tested, only the AP1/Ikaros mutant promoter showed a slight reduction in activity and the quadruple mutant promoter was just as active as the wild type promoter. None of the binding sites examined in this assay appeared to be important in L cells and it was apparent that all of these promoters were functioning in a non-specific manner. These results demonstrate that the

Figure 4-6

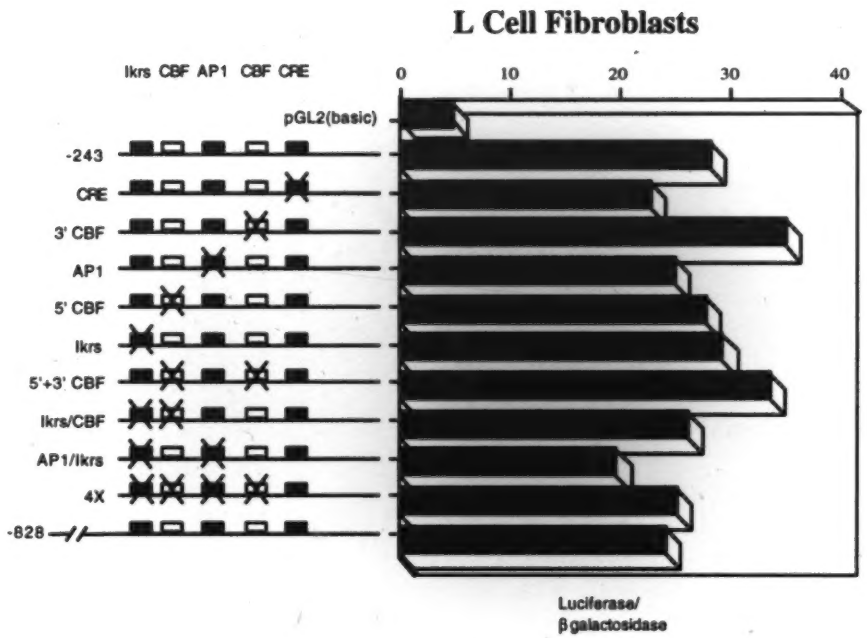


Figure 4-6 Expression of the granzyme B promoter mutants in murine L cell fibroblasts is insensitive to mutations.

The wild type and mutant promoter constructs were transfected into L cell fibroblasts and assayed for luciferase and β-galactosidase expression after two days. Values are depicted as luciferase/β-galactosidase.

negative effects of the mutations that we observed in T cells were relevant in that cell type.

DISCUSSION

The involvement of each of transcription factor in transcription of the granzyme B gene was assessed by abolishing their binding sites and measuring the resultant impact on promoter expression. Previous studies involving mutagenesis of the human granzyme B promoter have involved chemical stimulation of cell lines. In our studies, primary murine lymphocytes stimulated via the T cell and IL-2 receptors were utilized as the principal experimental system. Results from transfection studies in this physiologically relevant system were compared with data obtained from transfections of the same reporters in a T cell clone.

Primary lymphocytes express granzyme B mRNA in a stimulation-dependent manner and have a limited life span in culture. They require at least two independent signals for proliferation and CTL- specific gene expression. First, they are stimulated through the T cell receptor, by presented antigen, and second by IL-2 through the IL-2 receptor. MTL cells are different in that they are an antigen independent, IL-2-dependent, cytotoxic T cell line (Bleackley *et al.*, 1982). MTL 2.8.2 cells were selected for antigen independence by culturing in the presence of high levels of IL-2 and the phorbol ester PMA (Havele *et al.*, 1986), and they constitutively express all of the granzyme genes.

It was apparent from our previous transfection studies that reporter gene expression increased substantially as the length of the minimal promoter increased in length from 108 bp, to 169 bp, and to 243 bp. The shortest promoter included only the CRE binding site at -90. The next largest fragment included one CBF binding site at -125 and an AP1 at -150. The 243 bp promoter included another CBF binding site at -180 and Ikaros at -200. Promoter expression increased substantially in both primary lymphocytes and MTLs as more binding sites were appended, however, promoter activity decreased as the fragment

was extended to -402 bp. Therefore, our studies focused on the sites contained within the 243 bp fragment.

The granzyme B CRE is a target for a ubiquitous family of basic leucine zipper containing transcription factors (reviewed in Sassone-Corsi, 1995). These proteins may become potent transcriptional activators following phosphorylation through the cAMP stimulated protein kinase A signal transduction pathway. Stimulation through the T cell receptor results in increased levels of intracellular cAMP and the subsequent activation of transcription factors that are required for cAMP-responsive gene regulation. In the context of the short 108 bp promoter, the granzyme B CRE did not have significant transcriptional activation activity. However, it was quite important when included in the -243 promoter as its mutation reduced transcription to between 36% and 60% of wild type in T cells. Thus, CRE binding proteins only exerted a substantial positive effect when linked in *cis* with the other transcription factors.

The ubiquitous transcription factor AP1 is composed of heterodimers of the Fos and Jun family of DNA binding proteins. Upon T cell activation, Fos and Jun are induced by de novo synthesis and their transcriptional activation potentials are stimulated by phosphorylation (reviewed in Karin and Smeal, 1994). The DNA binding activity of AP1 is low or absent in resting T cells (Rincon and Flavell, 1994). Upon stimulation through the T cell receptor, these proteins were observed to form a strong specific complex with an oligonucleotide probe containing the granzyme B AP1 binding site and a distinct in vivo footprint (Babichuk *et al.*, 1996). Overall promoter expression in primary lymphocytes was lowered 73% to 61% of wild type when this site was altered in the -243 promoter. MTL cells were quite sensitive to mutations in the AP1 binding site (64% to 39% of wild type expression). The results with MTL are similar to studies involving the human granzyme B promoter where the AP1 binding site was found to be essential for reporter gene expression in TPA/bt2cAMP stimulated PEER cells (Hanson *et al.*, 1993). Analogously, the proximal AP1 binding site in the IL-2 gene promoter was necessary for

high levels of reporter gene expression in both PMA stimulated human peripheral blood cells and PMA stimulated Jurkat T cells (Hughes and Pober, 1996). The MTL line was developed from T cells treated with high levels of PMA and IL-2. The difference between our findings in primary cells and previous data in T cell clones may be explained by an inflated PKC response due to stimulation by phorbol esters.

Core binding factor, also known as polyoma enhancer binding protein (PEBP2), is a heterodimer of an evolutionarily conserved family of three α subunits and a single β subunit (Kamachi *et al.*, 1990; Satake *et al.*, 1995; Wang and Speck, 1992; Wang *et al.*, 1993; Zaiman *et al.*, 1995). Following stimulation of T cells, the β-CBF subunit is believed to translocate from the cytoplasm into the nucleus where it augments the DNA binding activity of a lymphoid-specific α-CBF subunit (Lu *et al.*, 1995). Weak CBF DNA binding activity was observed in resting T cells but a distinct complex was observed to bind to the granzyme B 3' CBF site in activated T cells. Although both CBF sites in the granzyme B promoter displayed prominent in vivo footprints in activated T cells, the mutation of either or both CBF sites had little or no negative effect on transcription in murine CTL's. This observation was in sharp contrast to the requirement for functional CBF binding sites in the enhancers of several other lymphoid-specific genes including IL-3 (Taylor *et al.*, 1996), CD36 (Armesilla *et al.*, 1996), the macrophage colony stimulating factor (M-CSF) receptor promoter (Zhang *et al.*, 1996), and the T cell receptor (TCR) genes α, β, γ and δ (Hernandez-Munain and Krangel, 1995; Hsiang *et al.*, 1993; Hsiang *et al.* 1995; Sun *et al.*, 1995; Wotton *et al.*, 1994).

Recently, Tanaka *et al.* discovered that the human equivalent of the CBFα2 protein, AML1, was specifically phosphorylated on two serine residues in response to the activation of the extracellular signal-regulated kinase (ERK) pathway. Phosphorylation of the AML1 protein potentiated transcriptional activation through PEBP2 binding sites in reporter gene assays (Tanaka *et al.*, 1996). *Drosophila* runt domain proteins, which are homologous to the CBF family, have been observed to bend the angle of linear DNA up to 61° (Golling *et*

al., 1996). We have not investigated whether CBF causes DNA bending in the granzyme B promoter, but its spacing in relation to the other binding sites may be of significance if CBF is involved in the formation of a specific three dimensional nucleoprotein complex that may be important for transcription in chromatin associated DNA.

Ikaros is a zinc finger DNA binding protein that is expressed throughout hemopoietic development and is essential for the differentiation of the lymphoid lineage of cells (Georgopoulos *et al.*, 1992; Georgopoulos *et al.*, 1994). This protein has been observed to bind in vitro to a non-consensus DNA binding site in the granzyme B promoter in both resting and activated CTL's. The murine granzyme B Ikaros element is very similar to those found in the human granzyme B promoter and the CD3δ promoter. Mutations introduced into the Ikaros binding sites of both of these promoters significantly abrogated their expression in T cells (Georgopoulos *et al.*, 1992; Warginier *et al.*, 1995), whereas no negative effect was observed for mutations in the murine granzyme B Ikaros element.

In theory, a promoter fragment in which the 5' CBF and Ikaros binding sites are abolished should only be as active as the 169 bp fragment, if they contained the same complement of functional binding sites. Similarly, a promoter in which the AP1, Ikaros, and both CBF binding sites are abolished should reflect only the activity observed from the 108 bp promoter if all the major transcription factor binding sites between -108 and -243 have successfully been abolished. In our primary cell transfection experiments the expression of the 5' CBF/Ikaros double mutant was identical to that of the -169 promoter. In MTL cells the same double mutant was less active than the 169 bp fragment, indicating that no other important binding sites that contribute to activation exist between -169 and -243. The expression of the 4X mutant promoter was identical to the -108 promoter in both primary cells and in MTLs. Therefore, we conclude that no transcription factors, other than the ones we have identified, are playing a significant role in the transcription of the minimal granzyme B promoter.

Primary lymphocytes must remain sensitive to a variety of signals so that inducible genes can be precisely controlled by subtly different stimuli. In contrast, cell lines are likely locked into invariably active signal transduction pathways. The most compelling differences between the results of transfection studies performed in primary cells and those conducted in cell clones is that several transcription factors, such as AP1, CRE and CBF, were not as important for transcription in primary cells as they have been reported to be under different conditions in cell lines (Hanson *et al.*, 1993; Taylor *et al.*, 1996: Wargnier *et al.*, 1995). This may reflect the removal from normal growth and transcriptional control mechanisms which is typical of immortalized cell lines. MTLs were more sensitive to mutations in the CRE and AP1 binding sites than primary cells likely because they possess highly active forms of CRE binding proteins and AP1 subunits that would increase the activity of the granzyme B promoter to levels far beyond its normal output. Hence, the removal of these factors from the promoter complex would significantly affect expression. Although the primary cell results are more likely to reflect what is occurring in vivo, we are still using polyclonal activators of T cells and could be missing the subtle effects that occur with presented antigen stimulation. This approach is not technically feasible in transfection experiments due to the already low reporter gene expression observed in these assays. The major consequences of some of these mutations were reflected in both cell types but the CTL line was differentially sensitive to certain single and double mutations as compared to primary lymphocytes.

The IL-2 promoter is regulated by a number of lymphoid restricted and ubiquitous transcription factors that exert very stringent cell-specific and activation-dependent requirements for IL-2 expression (Chen and Rothenberg, 1993; Jain *et al.*, 1992; Seibenlist *et al.*, 1986). Recently, striking differences regarding the regulation of this promoter were observed in transfection experiments performed in standard cell lines and in primary lymphocytes (Hughes and Pober, 1996). The effects of various binding site mutations were assessed and compared between transient transfection assays performed in

Jurkat T cells and primary lymphocytes, both activated with PMA + PHA. Several mutations, such as those in the distal NF-AT and the NF-AP1 (NF-AT/AP1) binding sites were far more inhibitory to transcription in Jurkats than they were in primary cells. These findings are in agreement with our results with the granzyme B promoter. Indeed, both studies suggest that transcriptional control in cell clones is inherently distinct from that in normal T cells, possibly owing to different combinations of nuclear factors existing in each cell type. Still, other binding sites such as AP1, NF-AT, NFκB, and pOCT did display strong functional importance in primary cell transfections with the AP1 mutation decreasing transcription to lower than 20% of wild type. Thus, IL-2 transcription is distinct from that of granzyme B in that IL-2 expression can be abolished by mutations in individual transcription factor binding sites, whereas granzyme B cannot.

The transfection results in L cells indicate that the effects of the mutations were in fact T cell-specific. Granzyme B promoter expression in L cells did not appear to be affected by any of the individual or multiple binding site mutations. Granzyme B transcription appeared to be non-specific as the 4X mutant promoter was equally as active as the full complement of binding sites present in the wild type promoter. A plausible explanation is that it was simply functioning as a loading site for the basal transcriptional machinery, which would generate significant luciferase activity in these highly metabolically active cells.

Mutation of Ikaros alone had either limited or no effect on reporter gene activity in T cells; however, the double mutant promoter, 5' CBF/Ikaros, exhibited a substantial reduction in transcriptional activity in MTL cells but not in primary cells. Ikaros exists as several distinct isoforms with variable DNA binding and transactivational activities (Georgopoulos, 1994; Hahm *et al.*, 1994; Molnár and Molnár *et al.*, 1996). A variation in the types of Ikaros proteins present in the different cell types could explain this discrepancy but this has yet to be determined.

A paradigm that has arisen in several promoters is that CBF functions with certain other transcription factors with juxtaposed binding sites, namely Act-1 in the IL-3 promoter, the CCAAT enhancer binding protein in the M-CSF receptor promoter, Ets-1 in the TCR β enhancer, and c-myb in the TCR δ enhancer. Similarly, Jun and Fos proteins have been discovered to interact with several transcription factors via protein-protein interactions. These interactions affect promoter function either positively or negatively depending on the factor. For example, AP1 represses activation by the glucocorticoid receptor (Schule, *et al.*, 1990; Yang-Yen *et al.*, 1990) and MyoD (Bengal *et al.*, 1992; Li *et al.*, 1992) whereas it cooperatively increases promoter activation by association with Ets-c (Noti *et al.*, 1996), GATA-2 (Kawana *et al.*, 1995), and the estrogen receptor (Gaub *et al.*, 1990) in certain genes. AP1 association with GATA-2 or the estrogen receptor occurs in the absence of DNA binding and tethering of both factors to the promoter complex occurs when either binding site is present. The 3' CBF and AP1 binding sites are separated by two helical turns of DNA and their in vitro footprints are separated by less than 5 bp, thus potentiating the probability of factor-factor interactions. Mutations in either binding site did not abrogate expression but together their ablation reduced expression of the granzyme B promoter to background in primary lymphocytes. This suggests that both factors together are an integral component of the granzyme B promoter/activator complex and may associate in a novel functional capacity. The nature of this potential interaction is currently being investigated.

These studies strongly suggest a functional interaction between the AP1 and CBF complexes. No other examples of interaction between these factors have been identified, but functional cooperativity between either AP1 or CBF and other transcription factors has been observed in many other promoters. In most instances, the binding sites for AP1 or CBF and another factor are separated by not more that 10 bp and both intact sites are required for optimal DNA binding by either factor. This spacing appears to be critical as insertions of 5 or 10 bp between binding sites destroy cooperative binding and function as

a unit. The granzyme B AP1 and 3' CBF sites are separated by 20 bp and both factors bind their respective elements independently of one another. The removal of either AP1 or CBF from the promoter complex has a limited negative effect but the removal of both abrogates expression. This could denote a form of protein-protein interaction whereby the proteins associate in the absence of DNA binding and only one binding site is required to tether both factors to the promoter. A similar situation is exemplified by the endothelin-1 promoter where the transcription factor GATA-2 potentiated AP1 activity despite the absence of a functional GATA binding site and vice-versa (Kawana *et al.*, 1995). Similarly, the AP1 and GATA proteins were found to associate in the absence of DNA. Alternatively, AP1 and CBF could serve as a docking site for an adapter protein that requires at least one factor bound to the promoter for association.

We have successfully identified all of the *cis*-acting sequences that are responsible for the high levels of promoter activity observed from the 243 bp minimal promoter. These are the CRE, AP1, 3' CBF, 5' CBF, and Ikaros elements. T cell clones transcriptionally regulate granzyme B promoter expression in a manner that deviates from primary lymphocytes. Clones are differentially sensitive to certain mutations and thus may not contain the same complement of transcription factors as primary cells. Although no single factor was necessary for expression, the combination of AP1 and CBF was essential for the granzyme B promoter. Further studies are required to delineate the nature of interplay between AP1 and CBF but it is clear from our studies that murine granzyme B expression is regulated by transcription factors acting in concert.

CHAPTER 5

GRANZYME B GENE AND PROTEIN EXPRESSION IN CD4$^+$ PRIMARY LYMPHOCYTES

INTRODUCTION

Humoral and cellular immunity are mediated by specialized cell types that are activated by bioactive peptides called cytokines. Several cytokines are secreted by activated T helper lymphocytes (Th) that constitute the second primary population of antigen-specific lymphocytes that develop in the thymus. Cytokines evoke and activate lymphoid-derived B cells, macrophages, mast cells, eosinophils, CD8$^+$ T cells, and CD4$^+$ T cells. Th cells secrete their own autocrine growth factors and the types of cytokines produced depend on the initial stimulus as well as cytokines secreted by other lymphocytes. There exists a functional dichotomy of two general classes of cytokine producing Th cells that develop at an infection site (reviewed by Abbas *et al.*, 1996). One subset (Th1) secretes interleukin-2 (IL-2), interferon-γ (IFN-γ), and tumour necrosis factor β (TNF-β) and the other subset (Th2) produces cytokines such as IL-4, IL-5, IL-6, and IL-13. Although the cytokine secretion pattern allows for the general classification of each subset, exceptions have been noted, making neither subset exclusive.

According to classical dogma, the role of CD4 glycoprotein bearing T cells is to produce cytokines. Alternatively, the role of CD8 glycoprotein bearing T cells is to destroy cells displaying foreign antigens. In contrast to CTL, Th cells recognize exogenously derived peptide antigens on the surface of hematopoietically-derived antigen presenting cells in the context of MHC class II proteins (Chein and Davis, 1993). Although poorly understood at the time, reports of MHC class II-restricted killing by CD4$^+$ clones were published in 1977 and 1981 (Swain *et al.*, 1981; Wagner *et al.*, 1977) and it was thought that this phenomenon was little more that an artifact of in vitro cell culture. Since then,

several independent investigators have provided convincing evidence that $CD4^+$ T cells are, in fact, cytotoxic by means of three distinct mechanisms. In one mechanism, killing of TNF sensitive cells is passively attained by the secretion of TNF in close proximity to the target (Schmidt *et al.*, 1986; Smyth, 1992). A second mechanism, demonstrated by several groups, is mediated by the induction of apoptosis in target cells via fas-fas ligand-mediated cytotoxicity (Erb *et al.*, 1990; Ju, 1991; Stalder, 1994). The third mechanism involves cytotoxicity by cell-cell contact followed by granule exocytosis and this process requires extracellular calcium (Lancki *et al.*, 1991; Miskovsky *et al.*, 1994; Smyth, 1992).

The potentiality of the perforin/granzyme mode of killing by $CD4^+$ T cells led several groups to investigate further the role of perforin and granzymes in T helper-mediated cytotoxicity. Very convincing evidence for a granule-mediated cytolytic mechanism was presented by Miskovsky *et al.* (1994) when they stimulated $CD4^+$ CTL clones with HIV envelope proteins. Perforin protein expression and distribution was examined by immunohistochemical staining of several vaccine induced clones, including those which were highly cytotoxic and those which were weak killers. A granular pattern of perforin staining was detectable in highly cytotoxic $CD4^+$ cell clones whereas weakly cytotoxic clones expressed fewer cells where perforin and granules were evident. Moreover, perforin mRNA was detectable by quantitative PCR in all vaccine induced clones and the amount of message tended to be proportional to the cytolytic activity of the cell.

In another report by Williams *et al.* (1996), the role of perforin in $CD4^+$-mediated cytotoxicity was investigated in primary cultures of murine lymphocytes stimulated for 5 days in the presence of irradiated allogenic spleen cells. The normally high cytolytic activity demonstrated by stimulated $CD4^+$ lymphocytes was dramatically inhibited in cultures derived from perforin knockout mice, indicating that perforin was an integral component of the cytolytic machinery. Similarly, Susskind *et al.* (1996) provided evidence for granzyme involvement in killing in that the cytolytic activity of certain antigen-specific $CD4^+$ cell clones could be blocked by the serine esterase inhibitor TLCK (Nα-*p*-tosyl-L-

lysine chloromethylketone) or with cytochalasin B and monensin (inhibitors of the secretory pathway). They also found that granzyme B and perforin transcripts were expressed in these cells and that they contained the granule associated protein TIA-1 (15 kd).

Physiologically, MHC class II-restricted cytotoxicity would destroy the very antigen presenting cells that activate $CD4^+$ T helper lymphocytes. Therefore, the expression of cytolytic genes would not be expected to be as active in this cell type as they are in primary, activated $CD8^+$ killer cells. It is unknown how or why Th cells develop this form of cytotoxicity or whether it has any physiological relevance. Granzyme B has been shown to be expressed at the mRNA level in some $CD4^+$ Th clones. Because these clones were derived from multifunctional parental cells, and have been subject to selective pressures in cell culture in response to various growth factors and cytokines, they have very variable phenotypes. Only certain lines express the gene and kill, while others do not. Therefore, they may not reflect the properties of true T helper cells. For this reason, it was important to study the expression of the granzyme B gene in primary $CD4^+$ T lymphocytes to determine whether this expression is a property of real T helper cells or an artifact of cell culture.

No studies have yet investigated whether the granzyme B gene is expressed in primary activated $CD4^+$ lymphocytes. If so, we expected it to be differentially regulated in $CD4^+$ and $CD8^+$ T cell subsets because of their inherently different functions. Following an encounter with an antigen presenting cell, a naive $CD4^+$ T cell becomes activated via the engagement of the T cell receptor, the CD4 co-receptor, and various accessory molecules. Cytoplasmic receptor domains incite the activation of several protein kinase and calcium-regulated signal transduction pathways. These pathways intersect in the nucleus in the form of activated transcription factors that ultimately induce the transcription of Th cell-specific genes. Although many of the events that elicit activation in $CD4^+$ and $CD8^+$ cells are similar, it is likely that very important differences exist. It was therefore of interest to

determine whether the granzyme B gene is expressed in response to similar stimuli and regulated by similar DNA sequences in primary activated $CD4^+$ cells and $CD8^+$ cells. Differences in gene or protein expression may reflect inherent differences between the two T cell types with respect to signal transduction to the nucleus, transcriptional regulation, post-transcriptional regulation, protein stability, or protein trafficking.

Primary murine lymphocytes were utilized as our principal experimental system in which to study and compare granzyme B regulation in $CD4^+$ and $CD8^+$ cells. These cells were stimulated through the TCR and IL-2 receptors with αCD3, con A, and IL-2. Several methods were employed to enrich or purify the T cell subsets. These include positive immunomagnetic selection, immunocolumn enrichment, and fluorescence activated cell sorting (FACS). Granzyme B transcript levels were determined and compared between cell types by Northern blot analysis. The sequences involved in the regulation of the endogenous granzyme B gene were characterized by DNase1 hypersensitivity analysis in resting and activated primary $CD4^+$ lymphocytes. Transient transfection analysis was employed to ascertain the relative activities of granzyme B promoter sequences in conferring expression of a reporter gene in the two cell types. Finally, granzyme B protein expression was examined in purified populations of activated primary $CD4^+$ and $CD8^+$ cells by Western blot analysis. Together, these experiments have identified the promoter sequences involved in the transcriptional regulation of the granzyme B gene in $CD4^+$ T cells and show that they are the same sequences utilized in $CD8^+$ cells. Interestingly, these experiments also indicate that granzyme B protein levels are differentially regulated in the two cell types.

RESULTS

The expression of granzyme B messenger RNA has been well documented in $CD8^+$ CTL, natural killer cells, and in some $CD4^+$ T cell clones. Although our main interest focused on granzyme B gene expression $CD8^+$ primary splenocytes, it was important to determine which sequences were involved in regulating granzyme B expression in $CD4^+$ cells. Moreover, it was of interest to probe the status of the endogenous granzyme B gene in resting and activated primary $CD4^+$ cells to determine where important regulatory sequences were positioned.

Primary $CD4^+$ T Cells Activated With Con A, αCD3, and IL-2 Express Granzyme B mRNA.

We first set out to determine whether granzyme B mRNA was expressed in freshly activated primary $CD4^+$ cells. Primary murine splenocytes were stimulated for three days in the presence of αCD3, con A, and IL-2. Aliquots of the splenocyte cell culture were then passed over either a CD4 or a CD8 immunocolumn. The immunocolumns typically yielded populations that were over 80% pure, with negligible contamination by the opposing T cell subset (< 2%). Highly enriched $CD4^+$ and $CD8^+$ cells were collected and lysed and total RNA was prepared for Northern blot analysis. The resultant Northern blot was probed with a radiolabeled murine granzyme B cDNA and autoradiography was performed.

The granzyme B transcript is readily detectable in both the $CD4^+$ and the $CD8^+$ lanes, depicted by an arrow in the Northern blot in figure 5-1. The transcript is approximately three fold more abundant in activated $CD8^+$ cells than it is in activated $CD4^+$ cells, as measured by phosphorimager quantitation analysis (data not shown). The Northern blot was also probed with a γ-actin cDNA to confirm equal RNA loading in each lane. This was the case as the actin transcript levels were equivalent in both lanes.

Figure 5-1

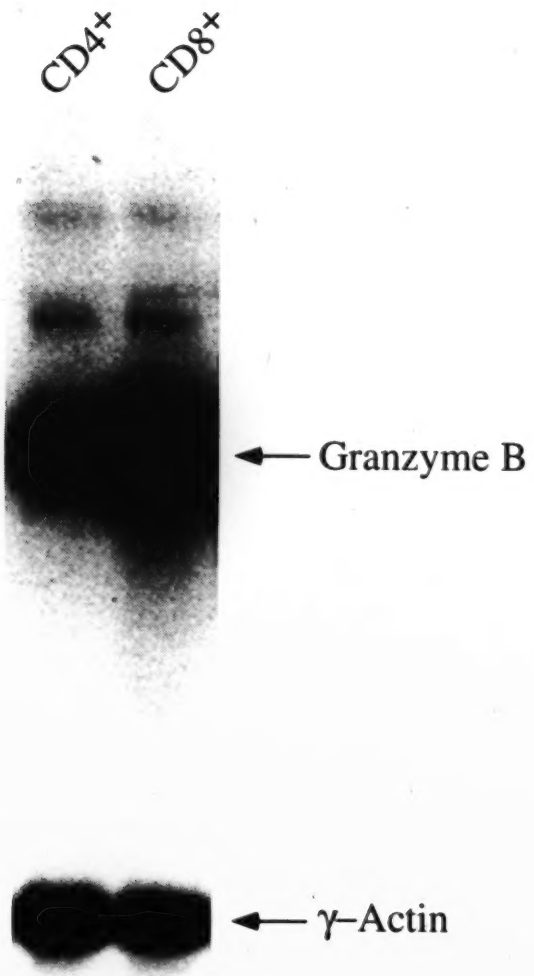


Figure 5-1 Northern blot analysis of the granzyme B mRNA in separated CD4+ and CD8+ primary lymphocytes.

CD8+ and CD4+ lymphocytes (stimulated for 3 days in the presence of IL-2, αCD3 and Con A) were isolated by passage over CD8 or CD4 immunocolumns. Total RNA was collected and 5 μg of each were separated on a 0.9% denaturing formaldehyde/agarose gel. The resulting blot was probed with a murine granzyme B cDNA and a human γ-actin cDNA.

The Proximal Promoter Sequences of the Endogenous Granzyme B Gene Display a Strong DNase1 Hypersensitive Site in Activated, But Not Resting, Primary CD4⁺ Lymphocytes.

To delineate further the exact sequences involved, DNase1 hypersensitivity analysis was employed to pinpoint the location of actively binding transcription factors on the endogenous promoter. Stimulated and unstimulated CD4⁺ cells were permeabilized by treatment with lysolecithin and the intact nuclear DNA was digested with increasing concentrations of DNase1. Hypersensitive areas in the granzyme B promoter could be detected within the 4.5 kb Eco RI fragment (discussed in Chapter 3) on a genomic Southern blot by end labeling this fragment with a probe that extends from -828 to -546 bp relative to the transcription start site.

Resting primary CD4⁺ lymphocytes were isolated by passing whole splenocytes over a CD4 immunocolumn and permeabilized cells were digested with various concentrations of DNase1. Following isolation and digestion of the genomic DNA with Eco RI, the samples were electrophoresed on an agarose gel and transferred to a nylon membrane by Southern blotting. The end labeled 4.5 kb Eco RI band in the resultant Southern blot in figure 5-2A gradually faded out with higher concentrations of DNase1. No regions of the fragment were hypersensitive to digestion by DNase1 as no specific sub-bands appeared. This result implied that the chromatin at the granzyme B locus was relatively condensed and that transcription factors were not binding to the promoter in resting CD4⁺ T lymphocytes. Activated CD4⁺ lymphocytes were then examined in the same manner. Unstimulated CD4⁺ and CD8⁺ splenocytes were isolated by passage over a T cell immunocolumn and were activated for three days in the presence of αCD3, con A, αCD28, and IL-2. The CD4⁺ population was removed from this culture by positive immunomagnetic separation and permeabilized cells were treated with various concentrations DNase1. The genomic DNA was isolated, digested with Eco RI and prepared on a Southern blot. The end labeled 4.5 kb Eco RI fragment in the resultant Southern blot in figure 5-2B gradually faded at

Figure 5-2. DNase1 hypersensitivity analysis in resting and activated CD4+ splenocytes shows that the granzyme B promoter is hypersensitive to DNase1 digestion in activated but not in resting cells.

Lysolecithin permeabilized cells were treated with between 0 and 10.0 μg/ml of DNase1. Genomic DNA was cut with Eco R1, separated on 1.2% agarose gels (15 μg/lane), and transferred to nylon membranes. The blots were probed with a 279 bp 5' granzyme B probe that indirectly end-labels a 4.5 kb Eco R1 fragment whose 5' end is 961 bp upstream of the first transcribed nucleotide.

A. The Southern blot of CD4+ unstimulated splenocytes does not indicate hypersensitive regions (6 day exposure; longer exposures of up to 2 weeks failed to reveal any hypersensitive sites). Resting $CD4^+$ lymphocytes were isolated from whole primary splenocytes by passage over a CD4 immunocolumn. $CD4^+$ cells comprised 82% of the enriched population and $CD8^+$ accounted for <1% as confirmed by FACS analysis.

B. Hypersensitive site formation in CD4+ splenocytes stimulated for three days with IL-2, αCD3, concanavalin A, and αCD28 (2 week exposure). Stimulated $CD4^+$ lymphocytes were isolated from a culture of mixed T cells (isolated by passage through a T cell immunocolumn) by immunomagnetic separation with Dynabeads™. The population is believed to be >98% pure. Size standards and the hypersensitive region are indicated to the right of the figure.

Figure 5-2

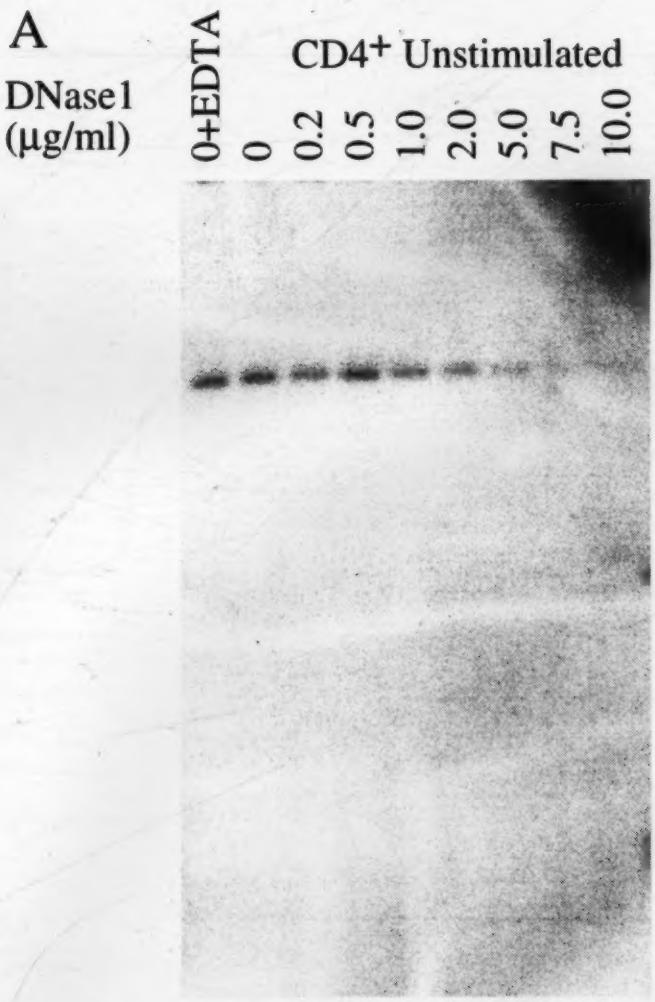
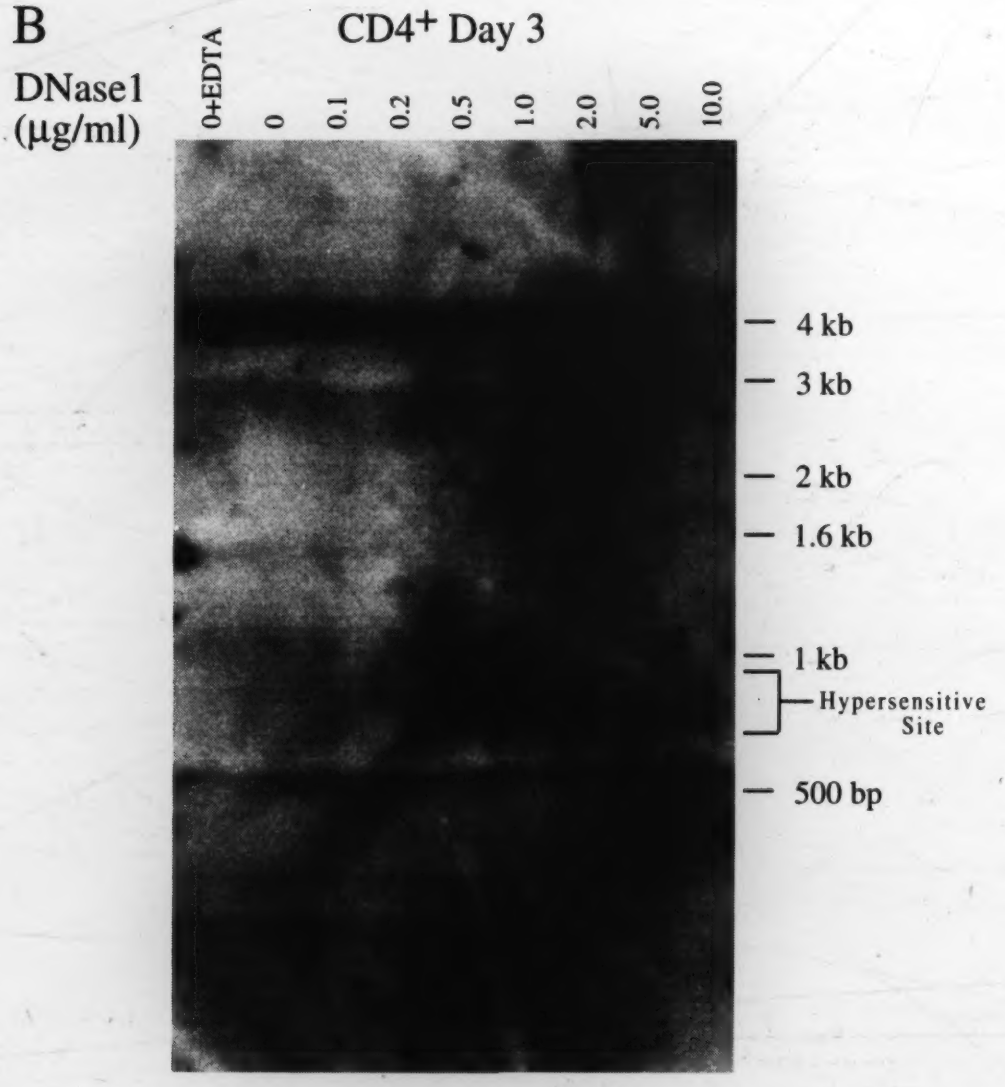


Figure 5-2


B
CD4+ Day 3
DNase1
(µg/ml)
0+EDTA
0
0.1
0.2
0.5
1.0
2.0
5.0
10.0
4 kb
3 kb
2 kb
1.6 kb
1 kb
Hypersensitive
Site
500 bp

higher concentrations of DNase1. Consequently, a sub-band of approximately 750 to 900 bp appeared that corresponds to the region encompassing the promoter sequences 50 to 200 bp upstream of the transcription start site in the granzyme B gene. This region contains the transcription factor binding sites CRE, AP1, CBF, and Ikaros. The presence of a hypersensitive site in this area indicates that these sites were being occupied by transcription factors in the endogenous gene and that it may be poised for activated transcription in activated $CD4^+$ primary lymphocytes. These experiments suggest that the endogenous granzyme B promoter undergoes an alteration in chromatin structure, that allows transcription factors access to the locus, and that this phenomenon is activation-dependent in primary $CD4^+$ lymphocytes.

Granzyme B Promoter Fragments Drive Reporter Gene Transcription in Activated Primary $CD4^+$ Lymphocytes in Transient Transfection Assays.

The results presented above indicate that 5' flanking sequences may be involved in the regulation of granzyme B gene expression in $CD4^+$ lymphocytes. To establish whether granzyme B promoter sequences were capable of driving transcription in activated $CD4^+$ lymphocytes, proximal promoter fragments were tested for the ability to express a reporter gene in transient transfection analysis. Three promoter fragments that extended from -108 to +29, -243 to +68, and -828 to +68 basepairs relative to the transcription start site were directionally subcloned into a promoterless luciferase reporter gene vector and were analyzed in transfection assays.

Whole primary murine splenocytes were stimulated in the presence of αCD3, con A, and IL-2 for 20 hours prior to transfection. The bulk cells were transfected with the reporter gene constructs and were then cultured for an additional two days under the same stimulation conditions. Transfected $CD4^+$ and $CD8^+$ T cells were isolated on day three post-initial stimulation (day two post-transfection). Aliquots of the whole cell cultures were incubated with either αCD4 or αCD8 antibodies prebound to secondary antibodies

Figure 5-3

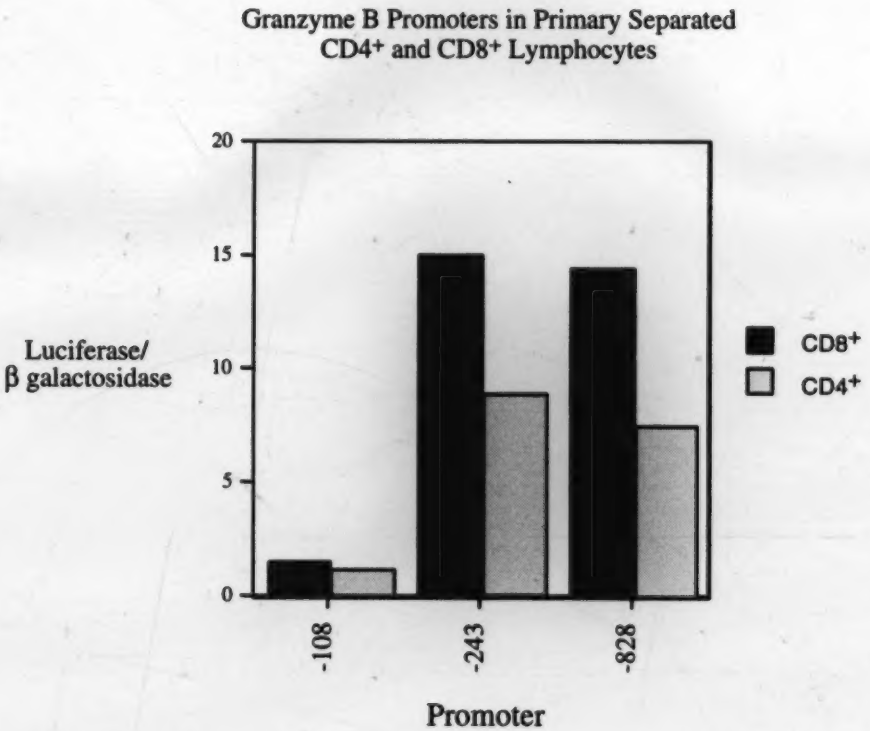


Figure 5-3 Transient transfection analysis of granzyme B promoter deletion fragments in CD4+ and CD8+ lymphocytes.

Three granzyme B promoter/luciferase deletion constructs (15 μg) were transfected into stimulated whole primary splenocytes. The CD4+ and CD8+ subsets were immunomagnetically isolated with Dynabeads two days following transfection (three days post-stimulation) for luciferase analysis.

that were conjugated to magnetic beads. The immunomagnetically conjugated cells were separated from the bulk culture by repeated magnetic separation and washing steps. The purified $CD4^+$ and $CD8^+$ cells were lysed and luciferase and β-galactosidase assays were performed.

The first two bars in Figure 5-3 depict the luciferase/β-galactosidase values for the granzyme B -108 promoter fragment. Its activity was not appreciable in either $CD8^+$ or $CD4^+$ cells and the values were essentially background for the assay. When the promoter fragment was extended 5' to -243, luciferase expression was significantly higher in both cell types (next two bars). The $CD4^+$ lymphocytes reported 59% of the luciferase activity observed in $CD8^+$ cells from this promoter. The results were very similar for the -828 promoter as it was essentially as active as the -243 promoter in both cell types (last two bars). It was expressed at relatively high levels in both cell types and $CD4^+$ cells reported 51% of the luciferase activity observed in $CD8^+$ lymphocytes. From these transfection studies we conclude that an important positive element exists in the granzyme B proximal promoter region between -108 and -243 and these sequences are responsible for the observed reporter gene expression in $CD4^+$ cells. Relatively, the promoter fragments were approximately half as transcriptionally active in $CD4^+$ T cells as they were in $CD8^+$ T cells.

Granzyme B Protein is Detectable by Western Blot Analysis in Activated Primary $CD4^+$ Lymphocytes.

The results presented above demonstrate that the granzyme B promoter was highly active in $CD4^+$ lymphocytes following stimulation for three days through the T cell and IL-2 receptors. Comparatively, the granzyme B mRNA was three fold less abundant in $CD4^+$ compared to $CD8^+$ cells. Therefore, it was of further interest to determine whether the granzyme B protein was also expressed to similar extents in each cell type. The relative levels of granzyme B protein in each cell type were then examined by Western blot analysis.

Figure 5-4

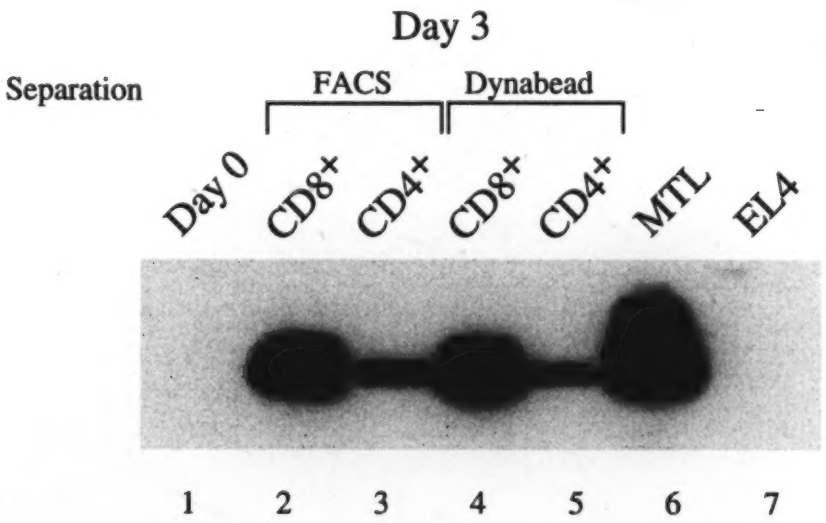


Figure 5-4 Western Blot analysis of granzyme B protein expression in purified stimulated CD4+ and CD8+ T cell subsets.

Whole primary lymphocytes were stimulated for three days in the presence of IL-2, con A and αCD3. CD4+ and CD8+ subsets were isolated by fluorescent activated cell sorting or by immunomagnetic separation. Cells lysates (30 µg each) from these cells plus lysates from unstimulated whole splenocytes, MTL 2.8.2 cells (positive control), and EL4 cells (negative control) were examined for the presence of the granzyme B protein by western blotting. The granzyme B protein was detected using a rabbit anti-mouse α-granzyme B primary monoclonal antibody, followed by a donkey anti-rabbit antibody conjugated to HRP. The immunocomplexes were visualized for 30 minutes using the ECL detection system.

Primary splenocytes were stimulated for three days in the presence of αCD3, con A, and IL-2. Purified $CD4^+$ and $CD8^+$ populations were obtained by positive immunomagnetic selection and by fluorescence activated cell sorting. The cell populations were collected and lysed and 30 μg of each lysate were separated on a denaturing SDS polyacrylamide gel. Protein was then electroblotted onto a polyvinylidene fluoride membrane and the granzyme B protein was detected using an αgranzyme B primary antibody. This antibody was then targeted using a horseradish peroxidase conjugated secondary antibody and the complex was detected using the ECL™ Western blotting detection system. The Western blot depicted in figure 5-4 shows that granzyme B protein was present in both activated $CD4^+$ and $CD8^+$ cells, isolated by either immunomagnetic separation (lanes 2 and 3) or by FACS (lanes 4 and 5). The protein was not detectable in unstimulated splenocytes (lane 1) or in EL4 thymoma cells (lane 7), but it was highly abundant in the MTL 2.8.2 cells that constitutively express the granzymes (lane 6). Interestingly, the protein was over 100 fold less abundant in $CD4^+$ cells than it was in $CD8^+$ cells. This result indicates that the granzyme B protein is not expressed in activated primary $CD4^+$ lymphocytes to nearly the same extent as it is in $CD8^+$ lymphocytes after three days of stimulation.

DISCUSSION

$CD4^+$ T lymphocytes have been found to destroy target cells by several mechanisms, one of which involves granule-mediated cytotoxicity. Upon receiving the appropriate stimulus, the T cell synthesizes and assembles the killing machinery de novo. Granzyme B is one gene that is activated in $CD4^+$ and $CD8^+$ lymphocytes upon activation and its protein constitutes an integral component of cytolytic granules. The regulation of granzyme B gene expression in activated $CD4^+$ lymphocytes was examined in this chapter by characterizing the promoter sequences that were necessary for the transcription of a reporter gene and those which were important in the endogenous gene. Overall, the transcriptional activity of

the granzyme B promoter was lower in $CD4^+$ compared to $CD8^+$ cells. As well, the gross discrepancy in protein quantity implies that distinct post transcriptional regulatory mechanisms exist in $CD4^+$ cells to downregulate the production or the accumulation of the granzyme B protein at times distinct from $CD8^+$ cells.

Northern blot analysis was employed to examine the relative accumulation of the granzyme B transcript in freshly stimulated $CD4^+$ and $CD8^+$ primary T cells. The granzyme B transcript was present in activated $CD4^+$ lymphocytes but the relative levels were three fold lower than those observed in $CD8^+$ lymphocytes. This discrepancy may either reflect differential activation of the cells by the stimuli received or differences in the kinetics of induction of the transcript (earlier or later peak). In a recent report by Williams *et al.* (1996) a 2.8 to 4.5 fold deficit of the perforin transcript was noted in $CD4^+$ splenocyte cultures compared to $CD8^+$ cultures. Interestingly, they found that $CD4^+$ cells were only capable of developing into MHC class II-specific CTL if the culture was depleted of NK cells and $CD8^+$ cells before stimulation with allogenic spleen cells. When stimulated in the presence of $CD8^+$ cells, killing by the $CD4^+$ cells was severely inhibited, and perforin transcript expression was reduced by 3.3 fold. It was suggested that this could represent a biological mechanism whereby $CD4^+$ CTL may develop as a backup for non-activated $CD8^+$ responses or when MHC class I molecules are lowered (Marusic-Galesic *et al.*, 1993; York *et al.*, 1994). In all of our experiments, $CD4^+$ and $CD8^+$ cells were stimulated together in the same culture. It is not known whether granzyme B expression in $CD4^+$ cells is affected by the presence of $CD8^+$ cells during stimulation but our results indicate that the gene is expressed at relatively high levels when both populations are stimulated together in the presence of αCD3, con A, and IL-2.

Major differences in granzyme B and perforin expression have been observed in various Th cell clones. Lancki *et al.* (1991) reported that most Th1 clones but not all Th2 clones could kill αCD3 coated target cells with varying degrees of efficiency. They found that only some of their Th2 clones expressed granzyme B and perforin mRNA, at levels

similar to those observed in CTL clones, whereas neither mRNA was detectable in Th1 clones. Alternatively, Hahn *et al.*, (1995) observed granzyme and perforin mRNA in all three of their cytolytic Th1 type clones and none in the two non-cytolytic Th2 clones tested. In another report by Hanson *et al.* (1991), certain clones of both the Th1 and Th2 subtypes were found to express the granzyme B transcript. Therefore, it appears that granzyme B expression is not limited to either subset of Th cells. Why only certain clones express the gene and kill while others do not remains unknown, but this could reflect the differences between clones and primary T lymphocytes. Different phenotypes and functions may become exaggerated or lost after selection in cell culture and they may not reflect the properties of the parental cells from which they were derived. Therefore, the importance of utilizing primary lymphocytes as a principal experimental system cannot be underemphasized.

DNase1 hypersensitivity analysis in permeabilized cells is a very useful tool that is used to locate regions of chromatin that are actively bound by transcription factors in vivo. A DNase1 hypersensitive site was observed in activated $CD4^+$ cells that was absent in resting cells. This indicates that the chromatin surrounding the granzyme B locus becomes decondensed in response to T cell activation. The hypersensitive region corresponds directly to the promoter sequences known to be important for transcriptional regulation of the granzyme B gene in activated $CD8^+$ lymphocytes. We can conclude from these studies that the cluster of transcription factor binding sites between -200 and -100 bp relative to the transcription start site are also involved in the regulation of the granzyme B gene in $CD4^+$ cells. Interestingly, the hypersensitive site was identical to the one observed in activated $CD8^+$ T cells (shown in Chapter 3, Figure 3-8B) in its size, location, and intensity. This suggests that the granzyme B locus was equally decondensed in both cell types. The strong intensity of the sub-band also implied that the majority of the population of $CD4^+$ cells was capable of expressing granzyme B in response to the stimulus issued. Interestingly, in $CD4^+$ cells from mice carrying a granzyme B promoter-regulated

transgene, the transgene RNA was expressed at much higher levels than the endogenous granzyme B mRNA (Hanson *et al.*, 1991). It was suggested that negative sequence elements existed outside of this region that may downregulate granzyme B expression in $CD4^+$ cells. While this may be true, the possibility exists that the transgene may have been as actively transcribed as the endogenous gene, but it was not subject to the same post-transcriptional regulatory mechanisms, such as mRNA degradation, as the granzyme B transcript.

In transient transfection assays, granzyme B promoter sequences were approximately half as active in $CD4^+$ cells as they were in $CD8^+$ cells, but the pattern of expression was very similar in both cell types. The short -108 promoter was inactive in both. It contained only the CRE binding site and the minimal transcription initiation sequences. Extending the promoter to -243 resulted in a dramatic increase in reporter gene expression in both $CD4^+$ and $CD8^+$ cells. The -828 promoter fragment was as active as the -243 promoter in both cell types, indicating that no additional positive elements exist between -243 and -828. Sequences between -108 and -243 were necessary for promoter expression in both $CD4^+$ and $CD8^+$ cells. This region contains five known binding sites for the transcription factors CRE, AP1, CBF, and Ikaros. All of these transcription factors should be present in $CD4^+$ T cells. CRE binding proteins are ubiquitously expressed, as are Fos and Jun family member proteins. The transcription factor Ikaros is restricted to the lymphoid lineage (Georgopoulos, *et al.*, 1992) and, although at least one α subunit and the β subunit of the CBF heterodimer are ubiquitously expressed, an α subunit is lymphoid-specific (Satake *et al.*, 1995). It is likely that very similar signal transduction events activate these transcription factors in both T cell subsets. These results show that the same sequences that were involved in granzyme B transcriptional regulation in $CD8^+$ cells are also important in $CD4^+$ cells.

After three days of stimulation, granzyme B protein expression was disproportionately low in $CD4^+$ compared to $CD8^+$ cells (<1%). This discrepancy may

reflect differential translational control or differences in protein stability. Either the mRNA was not being translated as efficiently or the protein was being degraded more rapidly in $CD4^+$ cells. No studies have yet been performed that investigate the half life of the granzyme B protein in $CD4^+$ or $CD8^+$ T cells. One report investigated the half-life of the cytolytic machinery in PMA + A23187 stimulated Th1 clones by treating the cells with the protein synthesis inhibitor cycloheximide (Ozdemirli *et al.*, 1992). The average half life, or the time required to deplete half of the cytotoxicity towards target cells, was a relatively quick 54 minutes. This study demonstrated that the cytolytic machinery was short lived in $CD4^+$ clones, but whether it was because of a highly unstable granzyme B protein is not known.

The studies described in this chapter have shown that the granzyme B gene is expressed in primary $CD4^+$ lymphocytes in response to activation and we have identified the major transcriptional controlling elements that are responsible for its induction. These are also the same sequences that were found to be important for granzyme B transcription in $CD8^+$ lymphocytes. Interestingly, we found that the quantity of the granzyme B protein in $CD4^+$ cells was not correlated with the level of expression of the endogenous gene, but was disproportionately low. It is likely that a mechanism of post transcriptional regulation that differs from $CD8^+$ cells is responsible for this discrepancy. This could reflect a mechanism, inherent in $CD4^+$ T cells, to downregulate the cytolytic machinery even if the genes are expressed at the mRNA level. This mechanism would be very important in vivo as it would prevent the unintentional destruction of antigen presenting cells by activated T helper lymphocytes, and allow the immune response to continue.

CHAPTER 6

SUMMARY AND GENERAL DISCUSSION

SUMMARY

Identification and Localization of Important *Cis*-Elements in the Murine Granzyme B Promoter

The sequences which are important for granzyme B transcription in activated cytotoxic T cells were investigated in a series of reporter gene, mobility shift, and DNase1 hypersensitivity assays, in addition to in vitro/vivo footprinting studies. It could be concluded from these experiments that granzyme B expression is regulated by at least four distinct transcription factors. These include members of the ubiquitously expressed AP1 and CREB/ATF transcription factor families, and the lymphoid-specific Ikaros and core binding factor proteins. Binding sites for these proteins were precisely localized by DNase1 footprinting studies and in vivo footprinting analyses. A CRE element exists at -90 bp relative to the transcription start site, followed by a CBF site at -125, an AP1 at -150, a second CBF site at -180, and Ikaros at -200 bp. These sequences are all contained within the 243 bp granzyme B promoter fragment, which was necessary and sufficient to activate high levels of reporter gene expression in T cell transfection assays.

Interactions between these transcription factors and their corresponding binding sites were investigated in resting and activated primary $CD8^+$ T cells. In resting T cells, Ikaros and a CBF subunit formed nucleoprotein complexes with their cognate sequence elements in vitro. However, no transcription factors were bound to the endogenous granzyme B promoter prior to stimulation. Following stimulation through the T cell and IL-2 receptors, AP1, CBF, and Ikaros formed strong in vitro nucleoprotein complexes and the endogenous granzyme B promoter was fully occupied at all five binding sites. As well, a DNase1 hypersensitive site was apparent in activated but not resting $CD8^+$ lymphocytes. This

implies that the granzyme B locus may not be accessible to transcription factors in resting T cells and that granzyme B induction involves decondensation at the chromosomal locus in response to T cell activation.

Mutational Analysis of Each Transcription Factor Binding Site

The importance of each transcription factor in the expression of the murine granzyme B gene was assessed by abolishing each binding site and measuring the resultant impact on promoter expression. It was found that a promoter fragment in which the AP1, Ikaros, and CBF binding sites were mutated, was expressed at background levels in transfection assays. While certain individual mutations, such as those in the CRE and AP1 elements, reduced promoter expression to some extent, none of the single binding site mutations resulted in a dramatic decrease in promoter activity. However, a double AP1/3'CBF mutant promoter was severely impeded in primary lymphocytes and this suggests that these two factors may be exhibiting a novel protein-protein interaction.

Stimulated primary lymphocytes and an IL-2-dependent T cell clone were utilized as the principal experimental systems in these transfection assays. Interestingly, the clones appeared to be differentially sensitive to certain single and double mutations, as compared to freshly activated lymphocytes. This study implies that transcriptional control in cell clones may be divergent from that in normal T cells and that they may possess a dissimilar complement of activated nuclear factors.

Granzyme B Expression in Primary $CD4^+$ Lymphocytes

Although $CD4^+$ lymphocytes are not believed to kill via the granule-mediated pathway in vivo, they were found to express the granzyme B transcript upon stimulation through the T cell and IL-2 receptors. The promoter sequences that are responsible for granzyme B transcription in this cell type were characterized by transient transfection assays and were localized between 108 and 243 bp upstream of the transcription start site. This region was

also hypersensitive to DNase1 digestion in activated, but not resting, $CD4^+$ lymphocytes. These sequences correspond directly those known to be important for granzyme B transcription in $CD8^+$ cells. Although the levels of granzyme B mRNA were only three fold lower in activated $CD4^+$ compared to $CD8^+$ lymphocytes, the relative protein levels differed by over a hundred fold. These studies suggest that while the granzyme B gene may be activated by the same complement of transcription factors as in $CD8^+$ cells, distinct post transcriptional regulatory mechanisms exist in $CD4^+$ lymphocytes which downregulate the expression of the granzyme B protein.

GENERAL DISCUSSION

Cytotoxic T cells constitute our main immunological defense against viral infections and tumours. The activation of CTL receptors by viral or tumour antigens results in the induction of several diverse signal transduction pathways, each of which regulate the activation of a particular subset of transcription factors. These factors then converge in the nucleus to activate the transcription of a specific subset of genes, which allow the CTL to acquire killing potential. By identifying the transcription factors that are involved in the T cell-specific expression of the granzyme B gene, we have successfully ascertained some of the molecular events that occur upon CTL activation (Figure 6-1).

The AP1 family of transcription factors is activated by several distinct, but overlapping, signal transduction pathways. Soon after T cell stimulation, Jun and Fos proteins are transcriptionally induced through the activation of the JAK/STAT, ERK/JNK, PKA, and calcium-dependent protein kinase pathways. These pathways target a set of transcription factors that bind to specific *cis*-acting response elements in jun and fos gene promoters (reviewed in Su and Karin, 1996). The newly synthesized AP1 proteins are activated by phosphorylation on serine/threonine residues in their transcriptional transactivation domains. Phosphorylation is mediated by JNK and FRK, which are activated by MAP kinases. In addition, JNK appears to be regulated by calcium-mediated

signals, which are activated in response to increased intracellular calcium concentrations. The removal of inhibitory phosphates from serine and threonine residues in the DNA binding domain of Jun stimulates its interaction with DNA and requires PKC dependent signals. Therefore, the involvement of AP1 in the transcriptional activation of granzyme B suggests that the protein tyrosine kinase, protein kinase C, and MAP kinase signal transduction pathways are engaged in activated CTL.

Although the molecular details of core binding factor activation are far from complete, it is apparent that ERK kinases are involved in at least one aspect (Tanaka *et al.*, 1996). The CBFα subunit is phosphorylated by ERK on two specific C-terminal serine residues, which have been shown to potentiate transcriptional transactivation by CBF. Interestingly, the CBFα C-terminal domain was also shown to be required for the nuclear translocation and association of the CBFβ subunit (Lu *et al.*, 1995). ERK-dependent phosphorylation of CBFα may augment its DNA binding and transcriptional transactivation potentials by permitting the nuclear translocation and association of the β subunit. Since CBF is essential for granzyme B expression in activated primary T cells, it is likely that the ERK/MAP kinase pathway is engaged.

The CRE binding proteins that target the granzyme B promoter were quite important when linked in *cis* with the other transcription factors. The activity of several CREB/ATF family members is mediated by phosphorylation through the cAMP-dependent protein kinase A pathway. Specifically, phosphorylation on serine 133 of CREB promotes the association of the adaptor protein CBP (CREB binding protein), which has been found to directly interact with the general transcription factor TFIIB (Kwok *et al.*, 1994). Because PKA activity appears to be important for the maximal expression of the granzyme B promoter, it is expected that CTL activation is accompanied by an increase in intracellular cAMP.

Figure 6-1 Model of Granzyme B Activation by T Cell Signal Transduction Pathways

The recognition of antigen on target cells induces a signal transduction cascade that results in the activation of several regulatory proteins, such as protein kinase C, protein kinase A, and MAP kinases. These proteins directly or indirectly activate the various transcription factors that are responsible for granzyme B expression. The transcription factors assemble on the granzyme B promoter and activate the transcriptional machinery.

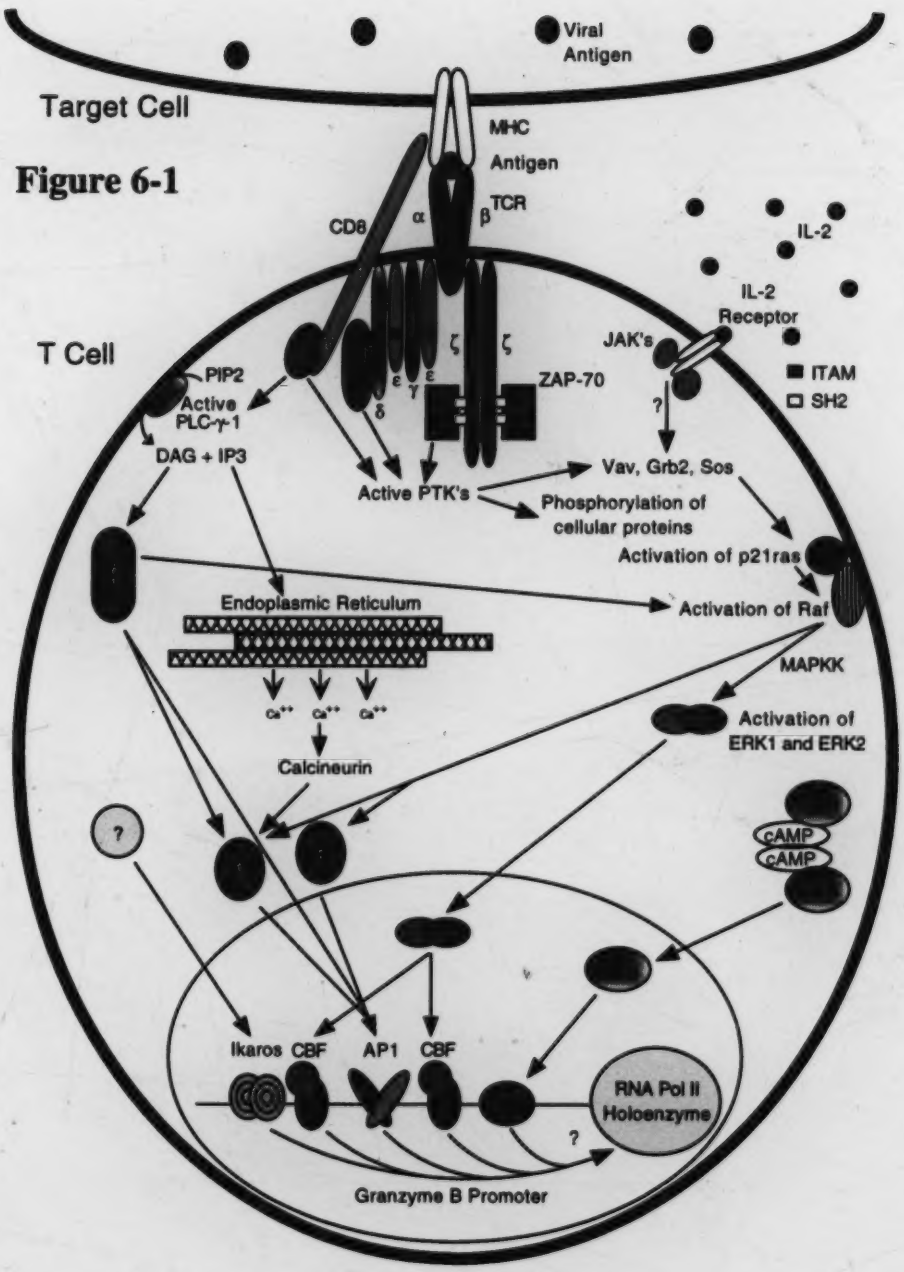

Viral
Antigen
Target Cell
MHC
Antigen
TCR
CD8
α
β
IL-2
IL-2
Receptor
T Cell
JAK's
ITAM
SH2
PIP2
Active
PLC-γ1
ζ
ζ
ε
γ
ε
δ
ZAP-70
?
DAG + IP3
Active PTK's
Vav, Grb2, Sos
Phosphorylation of
cellular proteins
Activation of p21ras
Endoplasmic Reticulum
Activation of Raf
MAPKK
Ca++
Ca++
Ca++
Activation of
ERK1 and ERK2
Calcineurin
?
cAMP
cAMP
Ikaros
CBF
AP1
CBF
RNA Pol II
Holoenzyme
?
Granzyme B Promoter

Figure 6-1

At this time, nothing is known about the mechanisms that control Ikaros activation. While Ikaros is present and capable of binding to DNA in resting lymphocytes, its transcriptional transactivation domain may require some form of protein modification. The most probable mechanism of Ikaros activation is phosphorylation. This possibility could be investigated by stimulating T cells in the presence of radioactive phosphorous and determining whether Ikaros becomes labeled in response to T cell activation.

There appears to be a considerable amount of overlap in the signal transduction pathways that control granzyme B gene expression. For example, treatment of cultured peripheral lymphocytes with high levels of IL-2 results in the induction of the granzyme B transcript (Liu *et al.*, 1989; Smyth *et al.*, 1990). This suggests that either TCR stimulation is not necessary for transcription, or that the IL-2 receptor is capable of transducing signals that overlap with TCR-mediated pathways. Recent evidence has implicated the cytokine receptor-associated JAK kinases as activators of the MAP kinase pathway. Although the mechanism of MAP kinase activation by JAKs is poorly understood, they appear to act upstream of p21ras, possibly through phosphotyrosine interactions with the Sos-linked adaptor proteins, Grb2 and Shc (reviewed in Winston and Hunter, 1996). As both CBF and AP1 are downstream targets of the MAP kinase pathway, and are essential components of the granzyme B enhancer complex, it is possible that they are sufficiently activated through the IL-2 receptor to induce granzyme B transcription in the absence of TCR-mediated signaling cascades. Alternatively, stimulation through the TCR induces the MAP kinase pathway and granzyme B expression in the absence of IL-2 stimulation. Thus, overlapping signal transduction pathways appear to make the role of IL-2 costimulation augmentative rather than imperative in CTL activation.

The proposed model of granzyme B transcriptional activation involves the integration of multiple signal transduction pathways that originate at the T cell and IL-2 receptors and terminate in the nucleus. Upon CTL/target cell interaction, these receptors initiate a protein tyrosine kinase cascade that secondarily activates the protein kinase C, MAP kinase, and

calcium-regulated pathways. Furthermore, TCR ligation results in an increase in intracellular cAMP concentration and the subsequent activation of protein kinase A proteins. The transcription factors AP1, CBF, Ikaros, and CREB/ATF are the nuclear targets of these pathways. Through various mechanisms of activation, these factors assemble in the nucleus and function synergistically in the displacement of a nucleosome from the granzyme B promoter. This event would either result in, or be the result of, a structural rearrangement of the chromatin at the granzyme B locus. The staged assembly of a higher-order protein complex on the enhancer would create the required three dimensional structure that would allow the transcriptional transactivation domains of the enhancer proteins to associate with the basal transcription machinery. Through various specific protein-protein interactions, these domains would activate the Pol II complex to initiate transcription and clear the granzyme B promoter.

The assembly of a precise multiprotein complex may be the molecular basis for the function of natural enhancers. For example, the specific arrangement of binding sites for the transcription factors CREB/ATF, LEF-1, CBF, and Ets-1 was very important in the TCRα enhancer (Giese *et al.*, 1995). Mutations, or alterations in the arrangement of these binding sites, disrupted enhancer activity in transfection assays. Interestingly, LEF-1 was found to be an essential architectural component of the complex. This protein induces a 130 degree bend in DNA molecule and its binding site is strategically positioned between the CRE and CBF/Ets-1 elements. This induced distortion is believed to facilitate direct contact between CREB/ATF and the CBF/Ets-1 proteins, as changes in the helical orientation of these binding sites (some 60 bp apart) dramatically reduced enhancer activity. Interestingly, LEF-1 activity could be functionally replaced by the SRY DNA bending protein. Therefore, architectural proteins, such as those that distort the DNA helix, appear to be very important components of enhancer complexes.

In a recent report by Golling, *et al.* (1996), the *Drosophila* runt proteins (homologs of mammalian CBF) were found to induce a 60 degree bend in a DNA fragment upon

sequence-specific binding. It is interesting to note that the 3' CBF element of the granzyme B promoter exists halfway between the AP1 and CRE binding sites (60 bp apart). Whether the structural properties of CBF are utilized to construct a specific three dimensional granzyme B enhancer configuration remains to be determined. This possibility could be investigated in the future by altering the spacing or the helical orientation of the binding sites and measuring the resultant impact on promoter expression.

Transfection assays demonstrated that the AP1 and CBF proteins play an essential role in granzyme B promoter expression. Further studies will be required to characterize the potential interaction between these two proteins and to elucidate their functional mechanism in vivo. This interaction appears to occur in the absence of DNA binding in vivo, as mutations in the individual AP1 and CBF elements did not significantly affect promoter activity. In a recent in vitro competition experiment, the trimolecular complex (AP1/CBF/DNA) was inhibited in the presence of excess AP1 or CBF oligonucleotides (B. Duggan, unpublished observations). As this protein-protein interaction may be unstable under the conditions of this binding assay, a direct association might still be observed by immunoprecipitation experiments. The generation of transgenic mice that carry these mutations within the endogenous promoter would determine conclusively whether AP1 and CBF are essential for granzyme B expression in vivo.

Chromosomally linked clusters of related genes, such as those of the globin locus, have been found to be controlled by a higher order of regulation, namely a locus control region (LCR) (reviewed in Felsenfeld, 1993). The human β-globin LCR consists of four developmental stage-specific DNase1 hypersensitive sites, that exist many kilobases upstream of the 5' most gene in the cluster, and control the sequential expression of the globin genes during development. (Frazer et al., 1993). The granzyme B locus consists of six structurally related granzyme genes that are expressed at various levels in response to T cell activation. Recently, considerable evidence has suggested that this locus is also controlled by an LCR. In a report by Pham *et al.* (1996), the replacement of a 350 bp

Avr II fragment in the murine granzyme B genomic gene (see Figure 3-7) with a PGK-Neo cassette (used to generate the granzyme B knockout mouse) abrogated the expression of several other granzyme genes within the cluster. This Avr II fragment contains part of the 5' untranslated region, exon 1, and part of intron 1. Within intron 1 there appears to exist potential binding sites for the transcription factors GATA-3, Ets, Myb, AP1, YY1, and STAT. However, no DNase1 hypersensitive sites exist in this region and the Avr II fragment did not display any enhancing effects in transient transfection assays when linked in *cis* to granzyme B promoter fragments (C. Babichuk, unpublished observations). It was suggested that the PGK-Neo cassette, which contains the constitutively active phosphoglycerate kinase promoter, disrupted the normal interactions between the putative granzyme LCR and the regulatory regions of distally located granzyme genes. A similar situation was exemplified in the β-globin locus where the PGK-NEO cassette disrupted the expression of several globin genes when inserted into one of the hypersensitive sites in the LCR. Upon removal, a much less severe phenotype was observed (Hug *et al.*, 1996). These findings suggest that the granzyme locus is controlled by an LCR and that it is either disrupted by the PGK-Neo cassette, or is replaced by it. The generation of transgenic mice in which the Avr II fragment is deleted would confirm the latter possibility. As LCRs usually manifest themselves as DNase1 hypersensitive sites, this type of assay could be utilized to search for such a site in distally located regions of the locus. The identification and characterization of a granzyme LCR would provide valuable information concerning the T cell-specific expression of the granzyme genes and an increased understanding of the higher order control of multigene loci.

Further studies will be required to investigate the mechanism of downregulation of the granzyme B protein in $CD4^+$ T cells. One possibility is that the granzyme B mRNA may be translated less efficiently in $CD4^+$ compared to $CD8^+$ cells. Alternatively, the protein could be either excreted or rapidly degraded in $CD4^+$ lymphocytes. The latter possibility could be investigated by pulse chase experiments to determine the half life of the

granzyme B protein in the two cell types. Preliminary experiments in our laboratory have suggested that the protein is not secreted, as no granzyme B activity was detectable in the supernatants of cultured $CD4^+$ lymphocytes. Understanding what mechanisms exist to prevent granzyme B protein accumulation in $CD4^+$ lymphocytes will provide valuable insights into the molecular distinctions between T helper and cytotoxic T cells.

The expression of exogenously derived genes in normal primary lymphocytes may have direct applications to gene therapy techniques. For this technique to be effective, an imported gene must be expressed in a cell-specific manner. The studies described in this thesis provide evidence that T cell-specific promoters can be introduced into peripheral lymphocytes and can be expressed at high levels in response to specific stimuli. The granzyme B promoter is a potentially valuable resource for use in gene therapy vectors. Therapeutic effects could be specifically targeted to T cells by the granzyme B promoter-directed activation of remedial genes, which would occur in response to T cell stimulation.

CONCLUDING REMARKS

Elucidation of the biochemical details of cellular activation has just begun. A considerable amount of progress in the signal transduction and transcription fields has led to a general understanding of how certain environmental stimuli effect cellular responses. Many more studies are required to illustrate the details of known mechanisms of activation, in addition to identifying previously uncharacterized ones. Moreover, deciphering the apparent crosstalk between signal transduction pathways will likely keep molecular biologists labouring for many years to come. A complete understanding of transcriptional activation in the context of nuclear chromatin will likely require many more years of research as the size and complexity of the transcriptional machinery is daunting. It will require a concerted effort by multiple multidiscipline laboratories, to elucidate the functional details of everything from the basic machinery to its higher order nuclear organization, to solve the complete structure of transcription.

CHAPTER 7

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APPENDIX I

TRANSCRIPTIONAL REGULATION AND MUTAGENESIS STUDIES OF THE SERPIN 2A PROMOTER IN T CELLS

INTRODUCTION

Hemopoietic progenitors of the bone marrow are self-renewing cells that are multipotent; that is that they give rise to several different lineages of blood cells. Mitotic divisions result in the generation of two distinct populations of daughter cells, those which remain multipotent and those which are developmentally destined to evolve into the erythrocytes, platelets, eosinophils, neutrophils, basophils, monocytes, B cells, or T cells. Dr. Ian Hampson (at the Paterson Institute of Cancer Research, Manchester, England) conducted a search for genes that may be involved in the differentiation of progenitor cells. Using differential cDNA library screening it was possible to identify genes that were expressed in the progenitor cells and then downregulated following differentiation (Hampson *et al.*, 1992). By this method they discovered that the granzyme B transcript is expressed in hemopoietic progenitors. Further investigation revealed that the granzyme B protein is also expressed in these cells. Following differentiation, mRNA and protein expression is turned off and neither reappear until fully mature T cells are activated through the T cell receptor by appropriate antigen recognition.

Another gene, serpin 2A, which encodes a serine proteinase inhibitor protein was found to maintain a very similar expression pattern to that of granzyme B. The complete cDNA of serpin 2A was cloned by differential cDNA library screening by comparing mRNA expression patterns between undifferentiated multipotent stem cells and those that were differentiated in vitro (Hampson *et al.*, 1997). The 2.3 kb transcript and 50 kD protein are highly expressed in the undifferentiated stem cells (Hampson *et al.*, 1997). The gene is downregulated over 250 fold at the level of transcription when hemopoietic

progenitors are induced to differentiate along the granulocyte-macrophage lineage in the presence of 1.5 U/ml IL-3, 50 U/ml granulocyte-macrophage colony-stimulating factor (GM-CSF), and 1000 U/ml granulocyte colony-stimulating factor (G-CSF). Granzyme B is also downregulated in the same manner, although to a lesser extent (six fold) by day six. The serpin 2A transcript was also detected in bipotent neutrophil/macrophage progenitors, isolated from bone marrow, but was downregulated when the cells were induced to differentiate along either the granulocyte (with G-CSF) or macrophage lineages (with M-CSF). A functional role for the serpin 2A protein has yet to be determined but other serpin proteins have been implicated in the differentiation of teratocarcinoma cells (Bielinska and Wilson, 1995; Wang, 1994), neuronal cells (Steele, *et al.*, 1993), muscle cells (Festoff *et al.*, 1990), and in enteric villus formation (Perlmutter *et al.*, 1989).

Like granzyme B, the serpin 2A mRNA and protein are strongly induced upon activation of mature T cells. Both transcripts and proteins are absent in quiescent splenocytes. Upon stimulation with 10 μg/ml con A and 60 U/ml IL-2, the serpin 2A mRNA level peaks after 24 hours and high levels persist for three days, whereas the granzyme B transcript peaks after four days and persists for several days (Hampson *et al.*, 1997). The serpin 2A protein is not detectable until day two but it is present for several days. Granzyme B expression is detectable on day one, it reaches peak levels within 4 days, and persists for several more. Most of the serpin 2A expression was found in the $CD8^+$ subset of primary T lymphocytes, while a reduced amount appeared in $CD4^+$ cells. Although these two genes share the same expression pattern with respect to activation dependence and cell type, the kinetics of induction and protein expression appear to differ temporally.

Because the pattern of serpin 2A expression corresponded with that of granzyme B, we were intrigued by the possibility that the two genes may be regulated by similar mechanisms, although there did not appear to be any obvious similarities between the promoter sequences. In collaboration with Dr. Ian Hampsons laboratory, we set out to

determine which sequences were responsible for the observed high levels of expression in T cells and which transcription factors were involved in the transcriptional regulation of the serpin 2A promoter.

RESULTS

Promoter Deletion Analysis Identified a Strong Positive Regulatory Element Between -127 and -258 Relative to the Transcription Start Site.

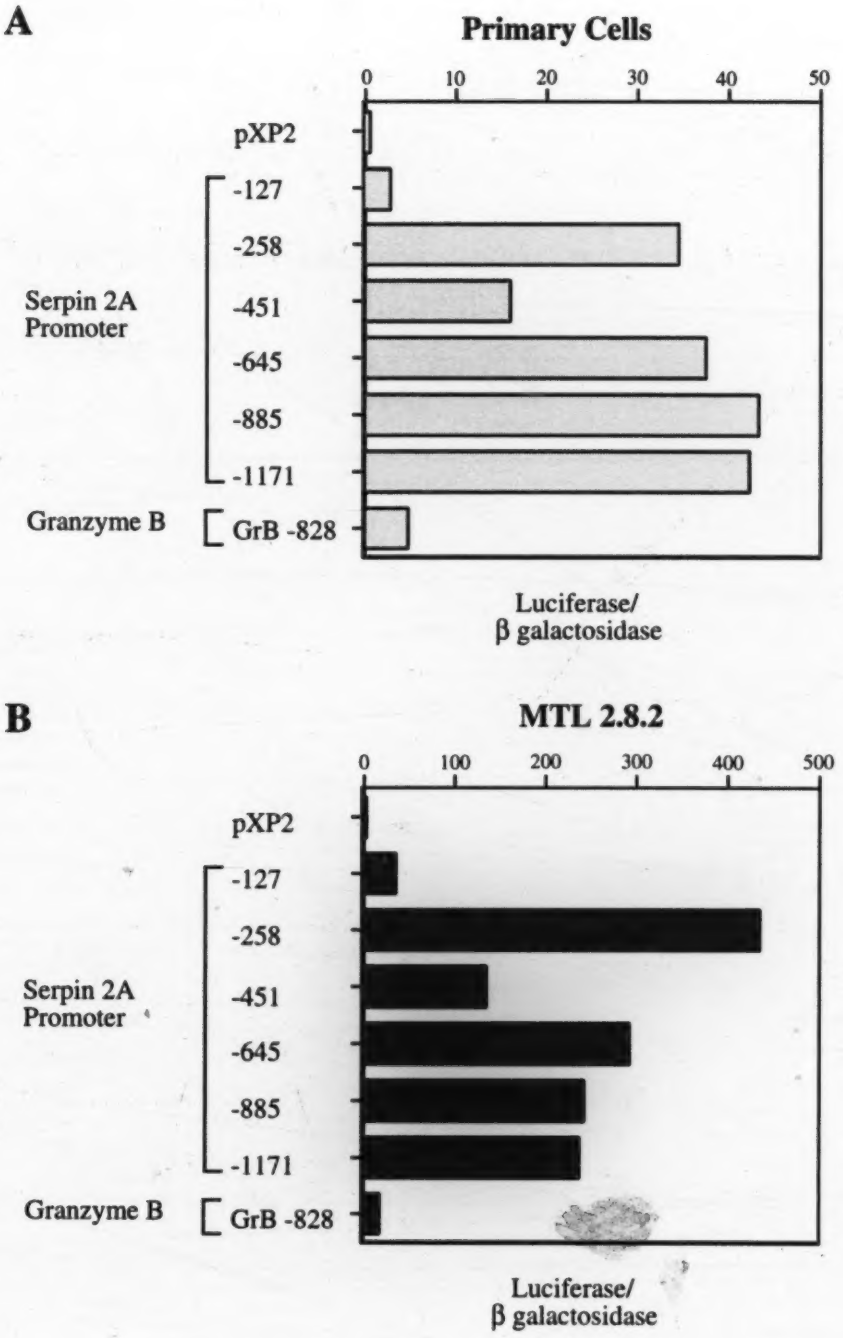
Six promoter deletion fragments were constructed by PCR amplification with the longest fragment extending 1171 bp upstream of the transcription start site. The fragments were directionally subcloned into a promoterless luciferase reporter gene vector and examined for reporter gene activity by transient transfection analysis in activated primary T lymphocytes and a $CD8^+$ T cell clone (MTL 2.8.2). The relative luciferase activities of the serpin 2A promoter deletion series in primary lymphocytes is depicted in Figure I-1A. The negative control in this experiment was the promoterless vector pXP2 and its luciferase expression was equal to background. The next six bars represent the transcriptional activity of the promoter fragments increasing in length from 127 bp to 1171 bp 5' of the transcription start site. The largest increase in expression, over twelve fold, occurred as the promoter was extended from -127 to -258. Sequences upstream of this area affected overall promoter activity minimally, inferring that no other major regulatory elements exist between -258 and -1171. The granzyme B -828 promoter, depicted by the next bar, is not as active in primary cells as its expression is nine fold lower than that of the full length serpin 2A promoter. This experiment indicates that a major enhancer region exists between -127 and -258 relative to the transcription start site.

The pattern of expression obtained by transient transfection analysis in the MTL 2.8.2 T cell clone is very similar to that in primary cells (Figure I-1B). The promoterless pXP2 vector was the negative control and its expression was background. The next six bars represent the same deletion fragments as above. The major increase in expression, over

Figure I-1 Promoter deletion analysis of the Serpin 2A promoter in stimulated primary lymphocytes and MTL 2.8.2 cells.

Six deletion fragments of the Serpin 2A promoter were cloned into the pXP2 promoterless luciferase reporter gene plasmid and were transfected (15 μg) along with an SVβ-galactosidase control plasmid (5μg) into **A.** primary lymphocytes and **B.** MTL 2.8.2 cells. Cells were harvested after two days and assayed for reporter gene activity. The values are depicted as the relative light unit value of the luciferase assays divided by the A420 value of the β-galactosidase assays. Promoter fragment lengths are shown on the left.

Figure I-1


A
Primary Cells
0
10
20
30
40
50
pXP2
-127
-258
-451
-645
-885
-1171
Serpin 2A Promoter
Granzyme B
GrB -828
Luciferase/
β galactosidase
B
MTL 2.8.2
0
100
200
300
400
500
pXP2
-127
-258
-451
-645
-885
-1171
Serpin 2A Promoter
Granzyme B
GrB -828
Luciferase/
β galactosidase

twelve fold, occurred as the serpin 2A promoter was extended from -127 to -258, analogous to the observed increase in primary cells. Similarly, no increase in expression was observed as the fragments were extended upstream to -1171. The 828 bp granzyme B promoter was over fifteen fold less active than the full length serpin 2A promoter in MTL cells. These results confirm that a powerful positive cis-element exists between -127 and -258 in the serpin 2A promoter that may be responsible for the high levels of reporter gene expression observed in MTL cells and in primary lymphocytes in transient transfection assays.

Mutational Analysis of Potentially Important Transcription Factor Binding Sites in the -127 to -258 Cis-Element

Once the major cis-enhancer element was localized to within 130 bp of DNA, potentially important transcription factor binding sites were identified using the transcription factor site search tool in the findpattern function of the GCG database search program. Three such regions were identified as being close matches to known transcription factor binding sites. These are comprised of an NFκB site at -179, a PEA3 site at -160, and an LVa site at -166. Each of these binding sites were abolished by site directed mutagenesis whereby three (NFκB), two (LVa), or one (PEA3) nucleotides were altered in the context of the -258 bp promoter fragment (Figure I-2). These three mutant promoters were transfected, along with the wild type promoter, into activated primary lymphocytes and MTL cells and assayed for luciferase reporter gene activity.

Figure I-3A depicts the results obtained when the mutant promoters were transfected into primary lymphocytes. The promoterless control vector pGL2 was utilized in this set of transfections and its expression was background. The wild type serpin 2A promoter was expressed at high levels as depicted by the second bar. The mutation of the NFκB binding site resulted in the most dramatic decrease in overall expression, which was 30% of wild

Figure I-2

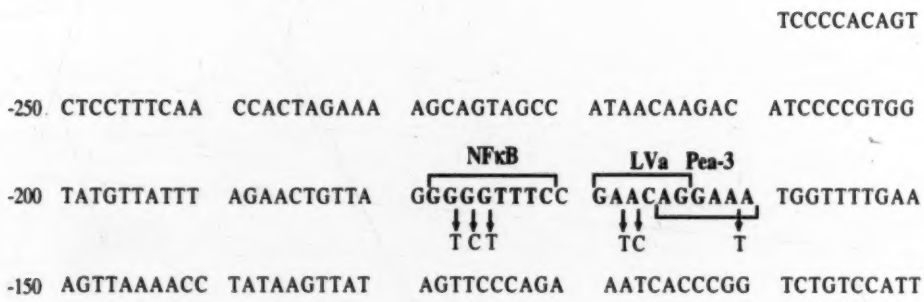


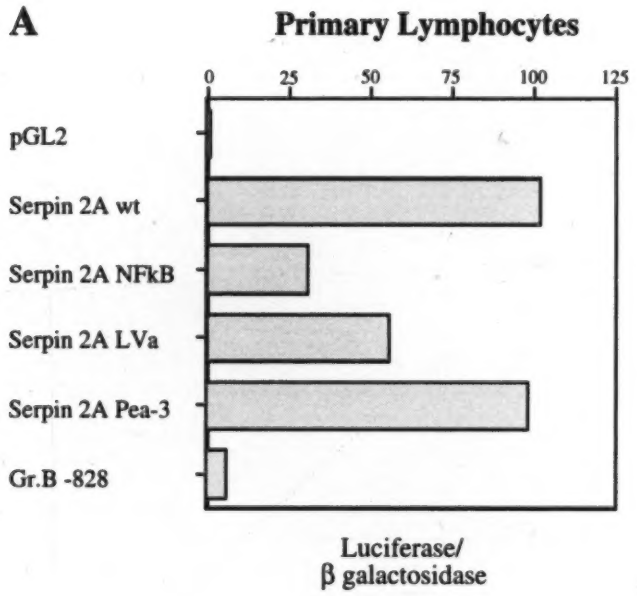
Figure I-2 The nucleotide sequence of the proximal Serpin 2A enhancer and the nucleotide substitutions present in the transcription factor binding sites.

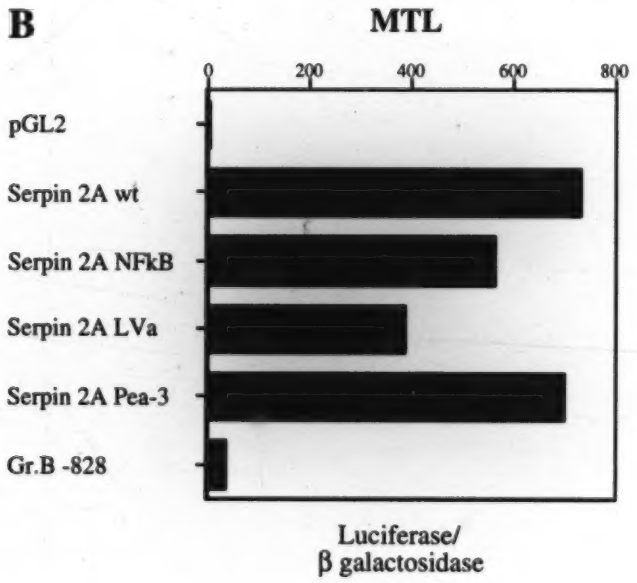
Major potential transcription factor binding sites are indicated in boldface and the transcription factor designations are shown above the sequences. Specific nucleotide substitutions are indicated by the arrows below the sequence for each binding site.

Figure I-3 Transient transfection analysis of Serpin 2A promoter mutations in stimulated primary lymphocytes and MTL 2.8.2 cells.

Three transcription factor binding site mutants of the Serpin 2A -275 bp promoter were cloned into the pXP2 promoterless luciferase reporter gene vector and were transfected (15 μg) along with an SVβ-galactosidase control plasmid (5 μg) into **A.** primary lymphocytes and **B.** MTL 2.8.2 cells. Cells were harvested after two days and assayed for reporter gene activity. The values are depicted as the relative light unit value of the luciferase assays divided by the A420 value of the β-galactosidase assays. The transcription factor binding site mutations in each fragment are indicated on the left.

Figure I-3


A
Primary Lymphocytes
0
25
50
75
100
125
pGL2
Serpin 2A wt
Serpin 2A NFkB
Serpin 2A LVa
Serpin 2A Pea-3
Gr.B -828
Luciferase/
β galactosidase


B
MTL
0
200
400
600
800
pGL2
Serpin 2A wt
Serpin 2A NFkB
Serpin 2A LVa
Serpin 2A Pea-3
Gr.B -828
Luciferase/
β galactosidase

type promoter. The LVa site mutation lowered expression to 55% of wild type (next bar) and the PEA3 mutation had no effect.

The same constructs as above were transfected into MTL 2.8.2 cells and the results are presented in figure I-3B. In contrast to the finding in primary cells, mutation of the NFκB sequence had a relatively minor effect on reporter gene activity in MTL, being 77% of wild type. The LVa site mutation reduced promoter expression to 53% of wild type and was very similar to the effect of the LVa mutation in primary cells. Similarly, the PEA3 binding site was dispensable to promoter activity in these assays. These mutagenesis studies indicate that NFκB and LVa are very important controlling factors in the regulation of transcription of the serpin 2A promoter. In addition, the results in primary cells did not reflect those obtained in MTLs as promoter expression was more sensitive to mutations in the NFκB element in primary lymphocytes than it was in MTL cells.

DISCUSSION

Transient transfection analysis was employed to determine the transcriptional activity of six promoter deletion fragments in primary activated lymphocytes and a T cell clone. It was determined that the major transcriptional enhancer of the serpin 2A promoter is located between -127 and -258 nucleotides relative to the transcription start site. This same region was also found to be important for promoter activity in transfection assays in undifferentiated stem cells (I. Hampson, personal communication). Sequence analysis and computer assisted pattern analysis within this region identified the potential transcription factors binding sites NFκB, LVa, and PEA3. By specifically abolishing each of these binding sites within the context of the 258 bp promoter fragment and transfecting these constructs into T cells we determined that the NFκB and LVa elements are important in serpin 2A gene regulation.

The ubiquitous transcription factor NFκB becomes a potent transcriptional activator within minutes following T cell activation (reviewed in Baldwin, 1996). Pre-existing

NFκB proteins are sequestered in the cytoplasm by a protein inhibitor, IκB. IκB is phosphorylated, and subsequently degraded, in response to TCR engagement. NFκB is released and is rapidly translocated into the nucleus where it may bind to its target DNA sequence and serve as a transcriptional activator. NFκB is clearly an important transcriptional activator of the serpin 2A gene. Mutations in this binding site lowered promoter activity to 30% of wild type in primary lymphocytes and 77% in MTLs. The results obtained in primary cells were very similar to those obtained in undifferentiated stem cell transfection experiments where it was observed that the mutation of the NFκB binding site reduced promoter expression to 25% of wild type (I. Hampson, personal communication). This discrepancy in values is likely reflective of the distinct characteristics of the cell types. Primary lymphocytes are freshly activated and possess all of the activated transcription factors that would be elicited during a natural T cell activation response whereas MTLs are transformed cell clones that likely do not posses the same compliment of specific transcription factors. The presence of NFκB in the promoter activation complex may explain the temporal difference in transcriptional induction between serpin 2A and granzyme B. Granzyme B transcription is presumably not regulated by NFκB as it is not sensitive to the inhibitory effects of the immunosupressant drug cyclosporin A, an inhibitor of NFκB activation (Frantz *et al*, 1994). As NFκB induction is an early event following T cell activation, it is possible that serpin 2A is targeted for activation long before granzyme B.

The mutation of the LVa binding site in the serpin 2A promoter resulted in a 50% reduction in activity in both primary lymphocytes and in MTL, and the same results were obtained in hematopoietic progenitor cell transfections (I. Hampson, personal communication). The LVa binding sequence was first characterized in the U3 region of the long terminal repeat of the Moloney murine leukemia virus. This sequence formed distinct complexes with nuclear extracts from a wide distribution of cell types and the two G residues were protected in methylation interference assays (Speck and Baltimore, 1987).

At this time, nothing is known about the nuclear protein(s) that interacts with this binding site.

Interestingly, the LVa site is juxtaposed to the NFκB site by only one nucleotide in the serpin 2A promoter. A functional interaction between these two proteins is not indicated in these experiments as mutations in either binding site reduced transcriptional activity to different extents. However, NFκB has been found to interact with several other transcription factors. These include the interferon regulatory factor-1 (IRF-1) and the high mobility group protein HMG-I(Y) in the vascular adhesion molecule-1 promoter (Neish *et al.*, 1995), the bZIP protein ATF-2 and HMG-I(Y) in the E-selectin promoter (Lewis *et al.*, 1994), and the Elf-1 protein and HMG-I(Y) in the IL-2 receptor α enhancer (John *et al.*, 1995). In the interferon β promoter, HMG-I(Y) was found to stimulate NFκB DNA binding (Thanos and Maniatis, 1995a). Thanos and Maniatis proposed that interactions between bZIP proteins (such as ATF-2 and Jun), IRF-1, HMG-I(Y), and NFκB proteins form higher order nucleoprotein complexes that are required for efficient activation of these enhancers (Thanos and Maniatis, 1995b). The LVa site does not resemble any of the binding sites discussed above. Therefore, further experiments are necessary to investigate the intriguing possibility of a novel interaction between LVa and NFκB in the serpin 2A promoter.

Other potentially important transcription factor binding sites were identified by computer analysis that may also be involved in the enhancer complex. For example, a consensus core binding factor sequence exists within the enhancer at -204 bp relative to the transcription start site. The presence of other transcription factor binding sites would be detected by future in vitro DNase 1 footprinting studies, to determine which promoter sequences bind to nuclear proteins, or by in vivo footprinting assays, to determine which sequences in the endogenous promoter are occupied by proteins during transcriptional activation.

The appearance of lymphoid specific transcripts in progenitor cells has been documented and may reflect a postulated means by which precursor cells 'prime' genetic loci for expression in mature lymphoid lineages. The TCRα and B220/CD45R transcripts are present in undifferentiated progenitor cells and the IgH gene enhancer is hypersensitive to DNase1, poising it for transcription (Ford *et al.*, 1992). It is possible that transcription of serpin 2A and granzyme B is reflective of this mechanism. It has yet to be determined whether transcription at this stage is necessary for expression in mature lymphoid lineages. Additionally, little is known about which regulatory sequences are involved in transcription in undifferentiated cells, however, it appears that they may be the same sequences that are important for the expression of the serpin 2A promoter in mature T cells. The pattern of expression of the deletion constructs and mutant promoters was identical in immature progenitor cells and primary activated lymphocytes (I. Hampson, personal communication). DNase1 hypersensitivity analysis or in vivo footprinting studies in these distinct cell types may resolve which sequence elements are utilized at the distinct developmental stages and could provide additional clues as to which transcription factors are involved in regulation.

At this time, nothing is known about the role of serpin 2A or granzyme B in the development of haematopoietic stem cells. It was established that both transcripts and protein products are expressed at high levels in these cells. As they are devoid of the characteristic cytotoxic granules and cytotoxic potential of T lymphocytes these proteins may possess an as yet unknown role in development. It is unknown whether these two proteins form a functional association with each other, one being a serine proteinase and the other being a serine proteinase inhibitor. A developmental role for granzyme B is not expected as granzyme B knockout mice develop normal numbers of lymphoid cells and their main phenotype is the reduced ability of CTL's to induce DNA fragmentation in target cells (Heusel *et al.*, 1994). Further studies, such as the generation of a serpin 2A knockout

mouse, are required for determining a possible role for the serpin 2A protein in hemopoietic development.

Although the serpin 2A and granzyme B genes appear to be expressed at similar times and in similar cells, it is clear from these transfection and mutagenesis studies that they are regulated by distinct mechanisms. The transcription factors NFκB and the LVa binding protein play a major role in serpin 2A transcription whereas neither appear to be necessary for the expression of granzyme B. This was not a surprising finding considering that many other T cell specific genes, which appear to be coordinately expressed in response to similar developmental or external stimuli, are controlled by different combinations of transcription factors. As more and more tissue specific enhancers are characterized, it is becoming apparent that no two promoters are identical and that the regulation of gene expression is a complex and subtly controlled process.

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